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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/724,108	12/01/2003	Hideki Thoda	245694US0CONT	3217
22850	7590	11/15/2006	EXAMINER	
C. IRVIN MCCLELLAND OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT, P.C. 1940 DUKE STREET ALEXANDRIA, VA 22314			SCHLAPKOHL, WALTER	
			ART UNIT	PAPER NUMBER
			1636	

DATE MAILED: 11/15/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/724,108	THODA ET AL.	
	Examiner	Art Unit	<i>W.S.</i>
	Walter Schlapkohl	1636	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) Responsive to communication(s) filed on 22 August 2006.

2a) This action is FINAL. 2b) This action is non-final.

3) Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

Disposition of Claims

4) Claim(s) 14-25 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5) Claim(s) _____ is/are allowed.

6) Claim(s) 14-25 is/are rejected.

7) Claim(s) _____ is/are objected to.

8) Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) The specification is objected to by the Examiner.

10) The drawing(s) filed on _____ is/are: a) accepted or b) objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11) The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12) Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a) All b) Some * c) None of:

1. Certified copies of the priority documents have been received.
2. Certified copies of the priority documents have been received in Application No. _____.
3. Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) Notice of References Cited (PTO-892)
2) Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date _____.

4) Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____.

5) Notice of Informal Patent Application

6) Other: Exhibit A.

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DETAILED ACTION

Receipt is acknowledged of the papers filed 8/22/2006 in which claims 1-13 were cancelled and claims 14-25 were added. Claims 14-25 are pending and under examination in the instant Office action.

Any rejection made in the previous Office action not recited herein is hereby WITHDRAWN.

Oath/Declaration

Receipt is acknowledged of the new Oath/Declaration filed on 8/22/2006. The new declaration is remedial and renders moot the objection to the oath/declaration recited in the previous Office action.

Specification

The disclosure is objected to because of the following informalities: the amendment to the specification filed 8/22/2006 in which the priority information was recited is objected to because 1) the amendment does not designate where the amended text should be inserted (lines numbers, but no page number, have been provided); and 2) the priority information appears to be incomplete, i.e., there is no reference to the

foreign priority document which is listed both on the originally filed ADS and the amended ADS filed 8/22/2006.

Also, the amended ADS filed 8/22/2006 is objected to because the amended ADS does not show the original incorrect information with strike-through. A supplemental ADS should show the changes made to ensure that the patent will reflect such change in accordance with MPEP 601.05 (section on Supplemental ADS Submissions).

Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 20, and therefore dependent claims 21-25, are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. **This is a new rejection necessitated by Applicant's amendment.**

Claim 20 recites "[a] method of producing a heterologous protein, comprising constructing a *Schizosaccharomyces pombe*

yeast cell which produces a heterologous protein by deleting or inactivating at least one gene encoding at least one enzyme selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase; and transforming the *Schizosaccharomyces pombe* yeast cell with a polynucleotide which encodes the heterologous protein" in lines 1-7 (emphasis added). Claim 20 is vague and indefinite because it is unclear whether Applicant intends construction of an *S. pombe* yeast cell in which the production of the heterologous protein would not occur without the deletion or inactivation of at least one gene encoding at least one enzyme selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase; or whether Applicant intends construction of an *S. pombe* cell for production of a heterologous protein comprising the steps of i) transforming the *S. pombe* cell with a polynucleotide which encodes the heterologous protein; and ii) deleting or inactivating at least one gene encoding at least one enzyme selected fro the group consisting of pyruvat decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase.

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The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 14-25 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. **This rejection is maintained for reasons of record but has been modified in order to accommodate Applicant's amendment.**

The claims are drawn to a method of constructing a *Schizosaccharomyces pombe* cell which produces a heterologous protein, comprising deleting or inactivating at least one gene encoding at least one enzyme selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase, and transforming the *S. pombe* yeast cell with a polynucleotide which encodes the heterologous protein wherein the deletion or inactivation of the at least one gene results in increased production of the

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heterologous protein compared to an *S. pombe* yeast cell in which the at least one gene has not been deleted or inactivated. The claims are also drawn to a method of producing and collecting the heterologous protein with such a cell. The claims encompass the production of any heterologous protein by any *S. pombe* host wherein any gene has been deleted or inactivated as long as at least a pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase and/or carboxypeptidase enzyme has been inactivated or deleted and wherein the deletion or inactivation of the at least one gene results in increased production of the heterologous protein. The claims do not provide any structural information with regard to the heterologous proteins which can be produced in combination with the deleted/inactivated genes, such that the deletion/inactivation results in increased production of the heterologous protein compared to a *Schizosaccharomyces pombe* in which the at least one gene has not been deleted or inactivated. Thus, the rejected claims comprise a set heterologous proteins and deleted/inactivated genes that are defined by their ability to result in improved protein production in an *S. pombe* yeast cell.

To provide adequate written description and evidence of possession of a claimed genus, the specification must provide sufficient distinguishing identifying characteristics of the

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genus. The factors to be considered include disclosure of a complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, and any combination thereof. The specification lists some genes which are unnecessary or detrimental to production of a heterologous protein in *S. pombe* such as pyruvate decarboxylase, aminopeptidase and carboxypeptidase (see, e.g. page 5, lines 20-27). The specification further lists yeast of the *Saccharomyces* genus and *Pichia* genus as preferable (see, e.g., page 6, lines 1-7). The specification also lists preferred examples of a number of genes considered by Applicant to be unnecessary or detrimental to production of the heterologous protein such as genes involved in energy metabolism (e.g., pyruvate decarboxylase which is involved in ethanol fermentation), genes associated with proteases such as endopeptidases and serine proteases. As examples of the instantly claimed invention, the specification describes *S. pombe* transformants which produce green fluorescent protein (GFP) efficiently by inactivation of a pyruvate decarboxylase gene, a serine protease gene, an aminopeptidase gene, a carboxypeptidase gene, an aspartic protease gene, a dipeptidyl aminopeptidase gene, a vacuolar carboxylase gene, a zinc protease gene, a metalloprotease gene, a CAAx prenyl protease

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gene, a dipeptidase gene, a methionine metallopeptidase gene, a methionine aminopeptidase gene, a signal peptidase gene and a mitochondrial processing peptidase gene. No description is provided of such a method or host, wherein the heterologous protein produced is any other than GFP. No description is provided of such a method or host wherein more than one gene has been inactivated or deleted.

Even if one accepts that the examples described in the specification meet the claim limitations of the rejected claims with regard to structure and function, the examples are only representative of one type of heterologous protein in the context of, at most, 13 classes or types of disrupted genes. The results are not necessarily predictive of any other eukaryotic microorganism host, any other heterologous protein or any other class of disrupted gene. Thus it is impossible to extrapolate from the examples described herein those heterologous proteins in combination with those deleted genes/enzymes that would necessarily meet the structural/functional characteristics of the rejected claims.

The prior art does not appear to offset the deficiencies of the instant specification in that it does not describe a set of eukaryotic microorganism hosts comprising deleted or inactivated genes or genomic regions such that there is improved production

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of a heterologous protein. A review by Giga-Hama and Kumagai describes the production of many heterologous proteins in *S. pombe*, but does not provide a single example of such a eukaryotic host in which a portion of the eukaryotic host's genome was deleted or inactivated in order to increase "the productivity of the heterologous protein" (*Biotechnology and Applied Biochemistry* 30:235-244, 1999).

Given the very large genus of heterologous proteins and the very large genus of deleted/inactivated genes encompassed by the claims, and given the limited description provided by the prior art and specification with regard to the combinations of heterologous proteins and deleted/inactivated genes capable of fulfilling the claim limitations of claims 14-25, the skilled artisan would not have been able to describe the broadly claimed genus of methods and proteins and deleted/disrupted genes such that any heterologous protein is produced and the production of the heterologous protein is improved over the *S. pombe* cell in which the recited genes have not been deleted/inactivated. Thus, there is no structural/functional basis provided by the prior art or instant specification for one of skill in the art to envision those hosts/heterologous proteins/deleted or inactive regions of the genome that satisfy the functional limitations of the claims. Therefore, the skilled artisan would

have reasonably concluded Applicant was not in possession of the claimed invention for claims 14-25.

Response to Arguments

Applicant argues that the rejection of claims 1-13 under 35 USC 112, first paragraph is no longer applicable in light of the cancellation of the claims. Applicant further argues that the issues raised in the previous Office action are not applicable to the newly presented claims. Applicant further argues that the yeast cell in the claims is limited to *S. pombe* and that the specific genes deleted or inactivated are listed. Applicant further concedes that although the examples in the specification utilize only a GFP marker protein as a model to show how the invention works, "once one has the modified yeast strain in hand, he/she can certainly transform that yeast cell with a heterologous polynucleotide sequence encoding a protein. Thus, the limitation to certain yeast cells coupled with the specific genes deleted or inactivated, as set forth on page 8-9 of the specification, demonstrates quite clearly that Applicants had possession of the invention as claimed" (see paragraph bridging pages 6 and 7 or the remarks filed 8/22/2006).

Applicant's arguments have been carefully considered and are respectfully found unpersuasive. While Applicant has gone

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some way to address the salient issues raised in the rejection of claims 1-13 under USC 112, first paragraph, raised in the previous Office action, the claims still contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. First, Applicant has not limited the *S. pombe* host to only those with specific gene deletions/inactivations; rather Applicant has limited the claims to include *S. pombe* yeast cells comprising deletions in one or more gene classes or types. In other words, Applicant has limited the claims to *S. pombe* cells comprising, for example, a deleted aspartic protease gene, but the claims are not specific with regard to the aspartic protease(s) which will suffice to meet the claim limitations. Furthermore, the specification only discloses one example of an aspartic protease used in the invention: the putative aspartic protease SPCC1795.09 (see pages 15-16). Moreover, while Examiner agrees with Applicant insofar as "once one has the modified yeast strain in hand, he/she can certainly transform that yeast cell with a heterologous polynucleotide sequence encoding a protein," this, in fact, is not the issue raised in the rejection recited in the previous Office action or in the instant Office action. The question

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here is whether Applicant has provided clear guidance in the way of examples in the specification and structural/functional information to convey to one of ordinary skill in the art that Applicant was in possession of the full scope of the claimed embodiments such that *S. pombe* cells with the appropriate deletions/incativations in at least one of the recited genes classes or types would yield increased production of any heterologous protein when transformed with DNA encoding the heterologous protein over the host's untransformed counterpart.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless -

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 14, 16, 20 and 22 are rejected under 35 U.S.C. 102(b) as being anticipated by Egel-Matani (US Patent No. 6,110,703). This rejection is maintained for reasons of record but has been slightly altered in order to accommodate Applicant's amendment(s).

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Note: for purposes of this rejection only, claim 20 has been interpreted as a method of producing a heterologous protein, comprising the steps of i) transforming an *S. pombe* cell with a polynucleotide which encodes the heterologous protein; ii) deleting or inactivating at least one gene encoding at least one enzyme selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase, wherein the deletion or inactivation of the at least one gene results in increased production of the heterologous protein compared to an *S. pombe* cell in which the at least one gene has not been deleted or inactivated, iii) culturing the yeast cell constructed such that the heterologous protein is produced by the yeast cell; and iv) collecting the heterologous protein.

Egel-Matani et al teach a method of constructing a transformed eukaryotic microorganism for the production of a heterologous polypeptide wherein the host comprises an inactive Yap3 protease which prevents the degradation of the heterologously produced protein (see entire document, especially column 19, claim 1; column 20, claim 11; as well as columns 10-12, Examples 6-10). Egel-Matani et al teach such a method wherein the eukaryotic host microorganism is *S. pombe* (see, e.g., column 4, lines 7-12; and column 20, claim 11). Egel-

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Matani et al further teach that inactivation of Yap3 results in 2-fold higher production of the polypeptide than would occur in a yeast with wild-type levels of Yap3 protease activity (column 19, claim 1). Egel-Matani et al also teach the collection/isolation of the heterologous protein (*ibid*).

Response to Arguments

Applicant argues that Egel-Matani describes deleting the YAP3 protease which "is a protease which cleaves arginine residues" (see page 7, 2nd full paragraph of the remarks filed 8/22/2006). Applicant further argues that "[w]hat Egel-Matani does not describe is the deletion of at least one gene chosen from pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase as set forth in the claims. Therefore, Applicant argues, the claims are not anticipated by the disclosure of this patent and as such withdrawal of the rejection is requested.

Applicant's arguments have been carefully considered and are respectfully found unpersuasive. Examiner notes for the record that the name YAP3 stands for yeast *aspartic protease 3* or yeast *aspartyl protease 3* as made clear in column 2, line 8, of the Egel-Matani reference. Examiner further directs Applicant to page 238 of the Stryer et al reference provided as

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Exhibit A (Biochemistry, 5th Edition, Chapter 9, pp. 227-260, Berg, Tymoczko, and Stryer, copyright 2002) in which is made clear that aspartic proteases function by utilizing two aspartate residues present within the enzyme's active site in coordination with a water molecule to cleave a peptide bond in a substrate protein. Therefore, Yap3's specificity for cleavage C-terminal to arginine residues is not indicative that Yap3 is NOT an aspartic protease.

Claims 14, 17, 19, 20, 23 and 25 are rejected under 35 U.S.C. 102(b) as being anticipated by Simeon et al (Yeast 11:271-282, 1996; IDS Ref. AW). **This rejection is maintained for reasons of record but has been slightly altered in order to accommodate Applicant's amendment(s).**

Note: for purposes of this rejection only, claim 20 has been interpreted as a method of producing a heterologous protein, comprising the steps of i) transforming an *S. pombe* cell with a polynucleotide which encodes the heterologous protein; ii) deleting or inactivating at least one gene encoding at least one enzyme selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase, wherein the deletion or inactivation of the at least one gene results in increased

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production of the heterologous protein compared to an *S. pombe* cell in which the at least one gene has not been deleted or inactivated, iii) culturing the yeast cell constructed such that the heterologous protein is produced by the yeast cell; and iv) collecting the heterologous protein.

Simeon et al teach a method of constructing a *Schizosaccharomyces pombe* eukaryotic host microorganism for the production of a heterologous protein, Carboxypeptidase Y of *Saccharomyces cerevisiae* (CY^{sc}) (see entire document, especially pages 271-272). Simeon et al teach such a method wherein the endogenous *S. pombe* Carboxypeptidase Y gene has been inactivated by exposure to ethylmethanesulfonate (EMS) and subsequent screening for *S. pombe* clones devoid of endogenous Carboxypeptidase Y (CY^{sp}) activity (see page 272, second column, first paragraph). Simeon et al teach the collection of the heterologous protein by sucrose density centrifugation (see page 277, Figure 3). Importantly, although Simeon et al do not explicitly teach such a method wherein the endogenous carboxypeptidase disruption results in improved production of the heterologous protein, such is the case inherently based upon Applicant's admission in the specification that "deletion or inactivation of part or all of the genome of the host unnecessary or detrimental to production of the heterologous

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protein by its transformant improves the production efficiency of the heterologous protein" (see instant specification at page 3, lines 24-27 and page 4, line 1). Because carboxypeptidase Y of is also a serine protease (see page 271, 2nd column wherein CYP was initially characterized as a serine exopeptidase), Simeon et al meet the claim limitations of claims 17 and 23.

Response to Arguments

Applicant argues that the Simeon publication is no longer applicable in light of the cancellation of the rejected claims. Applicant further argues that the Simeon publication is concerned with the characterization of the CPY^{sc} gene of *S. cerevisiae* in *S. pombe*, said gene encoding a serine exopeptidase. Applicant further argues that there is no discussion to specifically mutate at least one of a pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase and to express a heterologous protein at increased levels as claimed. Applicant further argues that contrary to the conclusion reached in the previous Office action, the expression of the *S. cerevisiae* gene in the mutant *S. pombe* strain does not inherently result in increased expression as shown in Table 2 on page 276 of the Simeon reference, wherein both wild type *S. cerevisiae* and *S.*

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pombe showed higher CPY activity than the mutant *S. pombe* containing the *S. cerevisiae* CPY gene.

Applicant's arguments have been carefully considered and are respectfully found unpersuasive. Simeon et al teach that the carboxypeptidase Y of *S. cerevisiae* (CPY^{sc}) is also known as PRC1 (see page 271, 2nd column, middle). The fact that CPY was "initially characterized as a serine exopeptidase" does not delimit the carboxypeptidase from the list of recited enzymes selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase; i.e. CPY^{sc} is a carboxypeptidase. With regard to Applicant's argument that the Simeon reference reaches a conclusion contrary to that set forth in the previous Office action, Applicant has improperly referenced data which were not used as the basis of the rejection. Applicant's claims are drawn toward the production of an *S. pombe* host comprising a deletion or inactivation of at least one enzyme selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase; and further transformed with a polynucleotide which encodes a heterologous protein, wherein the deletion or inactivation of the at least one gene results in increased production of the heterologous protein compared to a *Schizosaccharomyces pombe*

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yeast cell in which the at least one gene has not been deleted or inactivated. Applicant has referenced measurements of carboxypeptidase activity which is not indicative of heterologous protein expression compared to an *S. pombe* cell in which the at least one gene has not been deleted. Instead, Table 2 has merely been presented to show that the *S. cerevisiae* CYP expressed in the mutant *S. pombe* strain is enzymatically active. While the amount of carboxypeptidase activity in the transformed mutant *S. pombe* strain is lower than that of wild type *S. pombe*, this does not indicate that wild type *S. pombe* produces more CYP^{sc} because clearly untransformed wild type *S. pombe* does not produce any of the heterologous protein, CYP^{sc} (see Figure 1, page 275, lanes 2 and 3).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 14, 18, 20 and 24 are rejected under 35 U.S.C. 103(a) as being anticipated by Berka et al (WO 00/42203) in view of Giga-Hama et al (cited previously). **This is a new rejection necessitated by Applicant's amendment.**

Note: for purposes of this rejection only, claim 20 has been interpreted as a method of producing a heterologous protein, comprising the steps of i) transforming an *S. pombe* cell with a polynucleotide which encodes the heterologous protein; ii) deleting or inactivating at least one gene encoding at least one enzyme selected from the group consisting of pyruvate decarboxylase, aspartic protease, serine protease, aminopeptidase, and carboxypeptidase, wherein the deletion or inactivation of the at least one gene results in increased production of the heterologous protein compared to an *S. pombe* cell in which the at least one gene has not been deleted or inactivated, iii) culturing the yeast cell constructed such that the heterologous protein is produced by the yeast cell; and iv) collecting the heterologous protein.

Berka et al teach a method of constructing a yeast cell for the production of a heterologous protein wherein the yeast cell produces less cyclohexadepsipeptide than its parent cell (see entire document, especially the abstract). The yeast host may further comprise modifications which reduces or eliminates

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expression of proteins "that may be detrimental to the production, recovery, and/or application of the heterologous polypeptide of interest," including aminopeptidase (see paragraph bridging pages 8 and 9). Berka et al teach that this method can be performed with a *Schizosaccharomyces* yeast cell host (see, e.g., page 30, lines 13-15).

Berka et al do not teach the method performed specifically with an *S. pombe* yeast cell host.

Giga-Hama et al teach the use of *S. pombe* as a host for expression and production of foreign genes. Giga-Hama et al teach that there are several advantages to the use of *S. pombe* (see entire document, especially page 237, 2nd column, first paragraph). First, Giga-Hama teach that the *S. pombe* yeast has many characteristics more in common with higher mammalian cells than with other yeasts which makes this yeast host a good model for higher eukaryotic protein production and yields foreign gene products that are "closer to their natural form" (page 237, 2nd column, first-third paragraphs). Second, Giga-Hama et al teach that there are a number of vectors and promoters which result in efficient expression of a heterologous protein (see pages 239-240 and Table 1). Finally, Giga-Hama teach that *S. pombe* is a good host for the production of a number of different proteins (see, e.g., page 241, Table 2).

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It would have been obvious to combine the teachings of Berka et al with those of Giga-Hama et al because Berka et al teaches the expression of heterologous proteins in *Schizosaccharomyces* yeast cells and Giga-Hama teaches that *S. pombe* is a good yeast cell for foreign protein production.

One of ordinary skill in the art would have been motivated to combine the teachings of Berka et al and Giga-Hama et al because Giga-Hama et al teach that there are advantages of using the *S. pombe* cell, especially in cases where the heterologous gene to produces is a mammalian cell because the mammalian cell would be "closer to [its] nature form."

Based upon the teachings of the cited references, the high skill of one of ordinary skill in the art, and absent evidence to the contrary, there would have been a reasonable expectation of success to result when combining the teachings of Berka et al with those of Giga-Hama et al.

Conclusion

No claim is allowed.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL.** See MPEP § 706.07(a). Applicant is

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reminded of the extension of time policy as set forth in 37
CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Certain papers related to this application may be submitted to the Art Unit 1636 by facsimile transmission. The faxing of such papers must conform with the notices published in the Official Gazette, 1156 OG 61 (November 16, 1993) and 1157 OG 94 (December 28, 1993) (see 37 C.F.R. § 1.6(d)). The official fax telephone number for the Group is (571) 273-8300. Note: If Applicant does submit a paper by fax, the original signed copy should be retained by Applicant or Applicant's representative. NO DUPLICATE COPIES SHOULD BE SUBMITTED so as to avoid the processing of duplicate papers in the Office.

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Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to (571) 272-0547.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent applications to view the scanned images of their own application file folder(s) as well as general patent information available to the public.

For all other customer support, please call the USPTO Call Center (UCC) at (800) 786-9199.

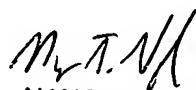
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Any inquiry concerning rejections or objections in this communication or earlier communications from the examiner should be directed to Walter Schlapkohl whose telephone number is (571) 272-4439. The examiner can normally be reached on Monday through Thursday from 8:30 AM to 6:00 PM. The examiner can also be reached on alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dr. Remy Yucel can be reached at (571) 272-0781.

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November 4, 2006


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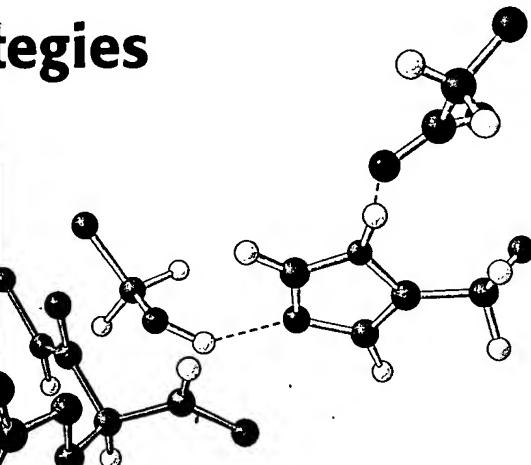
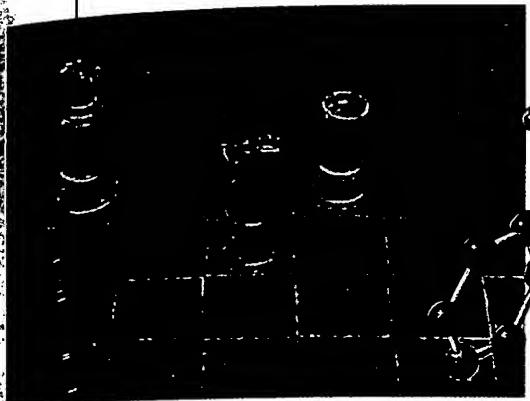
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Catalytic Strategies



Strategy and tactics. Chess and enzymes have in common the use of strategy, consciously thought out in the game of chess and selected by evolution for the action of an enzyme. The three amino acid residues at the right, denoted by the white bonds, constitute a catalytic triad found in the active site of a class of enzymes that cleave peptide bonds. The substrate, represented by the molecule with black bonds, is as hopelessly trapped as the king in the photograph of a chess match at the left and is sure to be cleaved. [(Left) Courtesy of Wendy Berg.]

What are the sources of the catalytic power and specificity of enzymes? This chapter presents the catalytic strategies used by four classes of enzymes: the serine proteases, carbonic anhydrases, restriction endonucleases, and nucleoside monophosphate (NMP) kinases. The first three classes of enzymes catalyze reactions that require the addition of water to a substrate. For the serine proteases, exemplified by chymotrypsin, the challenge is to promote a reaction that is almost immeasurably slow at neutral pH in the absence of a catalyst. For carbonic anhydrases, the challenge is to achieve a high absolute rate of reaction, suitable for integration with other rapid physiological processes. For restriction endonucleases such as EcoRV, the challenge is to attain a very high level of specificity. Finally, for NMP kinases, the challenge is to transfer a phosphoryl group from ATP to a nucleotide and not to water. The actions of these enzymes illustrate many important principles of catalysis. The mechanisms of these enzymes have been revealed through the use of incisive experimental probes, including the techniques of protein structure determination (Chapter 4) and site-directed mutagenesis (Chapter 6). These mechanisms include the use of binding energy and induced fit as well as several specific catalytic strategies. Properties common to an enzyme family reveal how their enzyme active sites have evolved and been refined. Structural and mechanistic comparisons of enzyme action are thus sources of insight into the evolutionary history of enzymes. These comparisons also reveal particularly effective solutions to biochemical problems that are used repeatedly in biological

OUTLINE

- 9.1 Proteases: Facilitating a Difficult Reaction
- 9.2 Carbonic Anhydrases: Making a Fast Reaction Faster
- 9.3 Restriction Enzymes: Performing Highly Specific DNA Cleavage Reactions
- 9.4 Nucleoside Monophosphate Kinases: Catalyzing Phosphoryl Group Exchange Without Promoting Hydrolysis

systems. In addition, our knowledge of catalytic strategies has been used to develop practical applications, including drugs that are potent and specific enzyme inhibitors. Finally, although we shall not consider catalytic RNA molecules (Section 28.4) explicitly in this chapter, the principles apply to these catalysts in addition to protein catalysts.

9.0.1 A Few Basic Catalytic Principles Are Used by Many Enzymes

In Chapter 8, we learned that enzymatic catalysis begins with substrate binding. The *binding energy* is the free energy released in the formation of a large number of weak interactions between the enzyme and the substrate. We can envision this binding energy as serving two purposes: it establishes substrate specificity and increases catalytic efficiency. Only the correct substrate can participate in most or all of the interactions with the enzyme and thus maximize binding energy, accounting for the exquisite substrate specificity exhibited by many enzymes. Furthermore, the full complement of such interactions is formed only when the substrate is in the transition state. Thus, interactions between the enzyme and the substrate not only favor substrate binding but stabilize the transition state, thereby lowering the activation energy. The binding energy can also promote structural changes in both the enzyme and the substrate that facilitate catalysis, a process referred to as *induced fit*.

Enzymes commonly employ one or more of the following strategies to catalyze specific reactions:

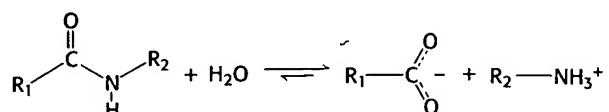
1. *Covalent catalysis.* In covalent catalysis, the active site contains a reactive group, usually a powerful nucleophile that becomes temporarily covalently modified in the course of catalysis. The proteolytic enzyme chymotrypsin provides an excellent example of this mechanism (Section 9.1.2).
2. *General acid-base catalysis.* In general acid-base catalysis, a molecule other than water plays the role of a proton donor or acceptor. Chymotrypsin uses a histidine residue as a base catalyst to enhance the nucleophilic power of serine (Section 9.1.3).
3. *Metal ion catalysis.* Metal ions can function catalytically in several ways. For instance, a metal ion may serve as an electrophilic catalyst, stabilizing a negative charge on a reaction intermediate. Alternatively, the metal ion may generate a nucleophile by increasing the acidity of a nearby molecule, such as water in the hydration of CO_2 by carbonic anhydrase (Section 9.2.2). Finally, the metal ion may bind to substrate, increasing the number of interactions with the enzyme and thus the binding energy. This strategy is used by NMP kinases (Section 9.4.2).
4. *Catalysis by approximation.* Many reactions include two distinct substrates. In such cases, the reaction rate may be considerably enhanced by bringing the two substrates together along a single binding surface on an enzyme. NMP kinases bring two nucleotides together to facilitate the transfer of a phosphoryl group from one nucleotide to the other (Section 9.4.3).

9.1 PROTEASES: FACILITATING A DIFFICULT REACTION

Protein turnover is an important process in living systems (Chapter 23). Proteins that have served their purpose must be degraded so that their constituent amino acids can be recycled for the synthesis of new proteins. Proteins ingested in the diet must be broken down into small peptides and

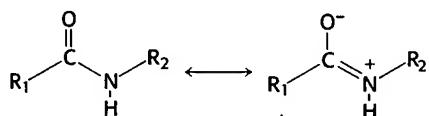
amino acids for absorption in the gut. Furthermore, as described in detail in Chapter 10, proteolytic reactions are important in regulating the activity of certain enzymes and other proteins.

Proteases cleave proteins by a hydrolysis reaction—the addition of a molecule of water to a peptide bond:



Although the hydrolysis of peptide bonds is thermodynamically favored, such hydrolysis reactions are extremely slow. In the absence of a catalyst, the half-life for the hydrolysis of a typical peptide at neutral pH is estimated to be between 10 and 1000 years. Yet, peptide bonds must be hydrolyzed within milliseconds in some biochemical processes.

The chemical bonding in peptide bonds is responsible for their kinetic stability. Specifically, the resonance structure that accounts for the planarity of a peptide bond (Section 3.2.2) also makes such bonds resistant to hydrolysis. This resonance structure endows the peptide bond with partial double-bond character:



The carbon–nitrogen bond is strengthened by its double-bond character, and the carbonyl carbon atom is less electrophilic and less susceptible to nucleophilic attack than are the carbonyl carbon atoms in compounds such as carboxylate esters. Consequently, to promote peptide-bond cleavage, an enzyme must facilitate nucleophilic attack at a normally unreactive carbonyl group.

9.1.1 Chymotrypsin Possesses a Highly Reactive Serine Residue

A number of proteolytic enzymes participate in the breakdown of proteins in the digestive systems of mammals and other organisms. One such enzyme, chymotrypsin, cleaves peptide bonds selectively on the carboxyl-terminal side of the large hydrophobic amino acids such as tryptophan, tyrosine, phenylalanine, and methionine (Figure 9.1). Chymotrypsin is a good example of the use of *covalent modification* as a catalytic strategy. The enzyme employs a powerful nucleophile to attack the unreactive carbonyl group of the substrate. This nucleophile becomes covalently attached to the substrate briefly in the course of catalysis.

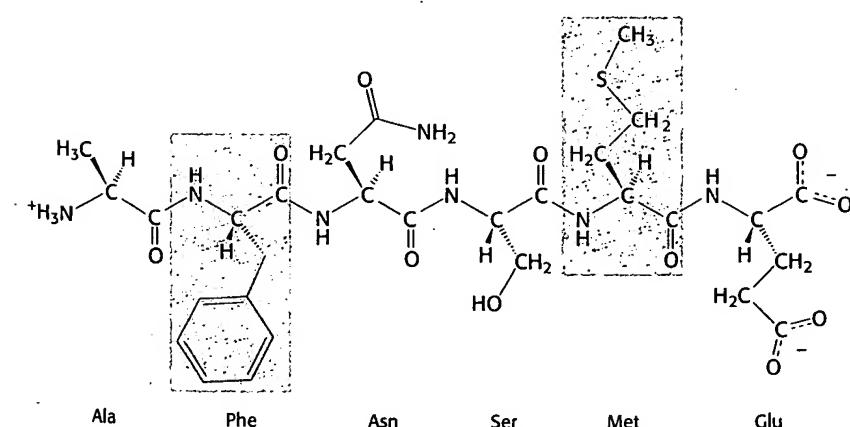


FIGURE 9.1 Specificity of chymotrypsin.
Chymotrypsin cleaves proteins on the carboxyl side of aromatic or large hydrophobic amino acids (shaded yellow). The likely bonds cleaved by chymotrypsin are indicated in red.

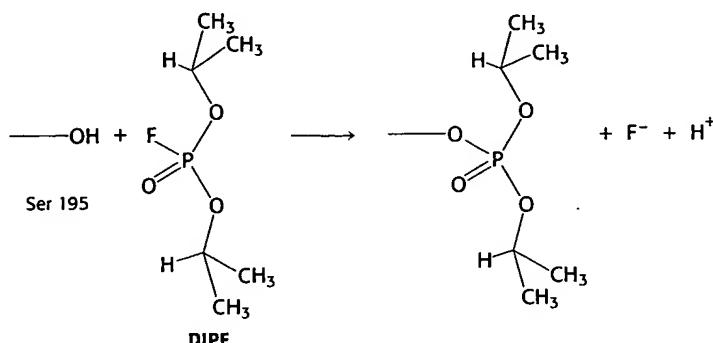


FIGURE 9.2 An unusually reactive serine in chymotrypsin. Chymotrypsin is inactivated by treatment with diisopropylphosphofluoridate (DIPF), which reacts only with serine 195 among 28 possible serine residues.

What is the nucleophile that chymotrypsin employs to attack the substrate carbonyl group? A clue came from the fact that chymotrypsin contains an extraordinarily reactive serine residue. Treatment with organofluorophosphates such as diisopropylphosphofluoridate (DIPF) (Section 8.5.2) was found to inactivate the enzyme irreversibly (Figure 9.2). Despite the fact that the enzyme possesses 28 serine residues, only one, serine 195, was modified, resulting in a total loss of enzyme activity. This *chemical modification reaction* suggested that this unusually reactive serine residue plays a central role in the catalytic mechanism of chymotrypsin.

9.1.2 Chymotrypsin Action Proceeds in Two Steps Linked by a Covalently Bound Intermediate

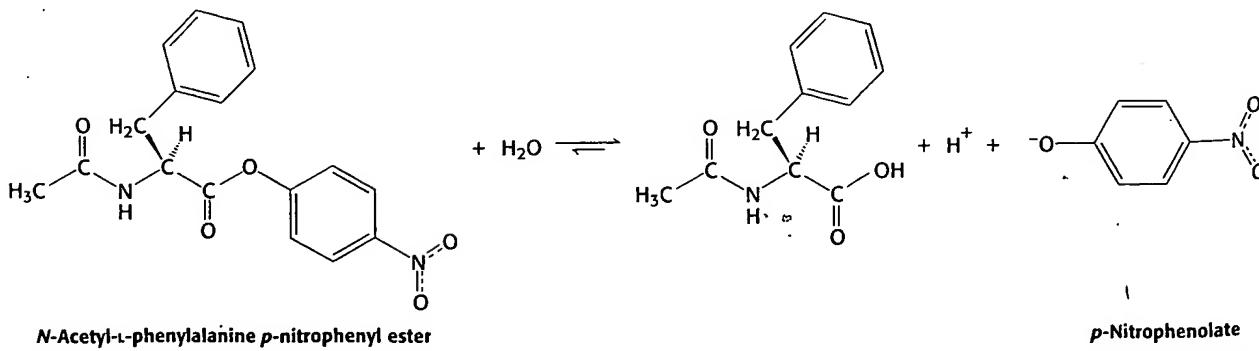


CONCEPTUAL INSIGHTS, Enzyme Kinetics. See the section entitled “Pre-Steady-State Kinetics” in the Conceptual Insights module to better understand why a “burst” phase at short reaction times implies the existence of an enzyme-substrate intermediate.

How can we elucidate the role of serine 195 in chymotrypsin action? A study of the enzyme’s kinetics provided a second clue to chymotrypsin’s catalytic mechanism and the role of serine 195. The kinetics of enzyme action are often easily monitored by having the enzyme act on a substrate analog that forms a colored product. For chymotrypsin, such a *chromogenic substrate* is *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester. This substrate is an ester rather than an amide, but many proteases will also hydrolyze esters. One of the products formed by chymotrypsin’s cleavage of this substrate is *p*-nitrophenolate, which has a yellow color (Figure 9.3). Measurements of the absorbance of light revealed the amount of *p*-nitrophenolate being produced.

Under steady-state conditions, the cleavage of this substrate obeys Michaelis-Menten kinetics with a K_M of 20 μM and a k_{cat} of 77 s^{-1} . The initial phase of the reaction was examined by using the stopped-flow method. This technique permits the rapid mixing of enzyme and substrate and allows almost instantaneous monitoring of the reaction. At the begin-

FIGURE 9.3 Chromogenic substrate. *N*-Acetyl-L-phenylalanine *p*-nitrophenyl ester yields a yellow product, *p*-nitrophenolate, on cleavage by chymotrypsin. *p*-Nitrophenolate forms by deprotonation of *p*-nitrophenol at pH 7.



ning of the reaction, this method revealed a “burst” phase during which the colored product was produced rapidly (Figure 9.4). Product was then produced more slowly as the reaction reached the steady state. These results suggest that hydrolysis proceeds in two steps. The burst is observed because, for this substrate, the first step is more rapid than the second step.

The two steps are explained by the reaction of the serine nucleophile with the substrate to form the covalently bound enzyme–substrate intermediate (Figure 9.5). First, the highly reactive serine 195 hydroxyl group attacks the carbonyl group of the substrate to form the acyl-enzyme intermediate, releasing the alcohol *p*-nitrophenol (or an amine if the substrate is an amide rather than an ester). Second, the acyl-enzyme intermediate is hydrolyzed to release the carboxylic acid component of the substrate and regenerate the free enzyme. Thus, *p*-nitrophenolate is produced rapidly on the addition of the substrate as the acyl-enzyme intermediate is formed, but it takes longer for the enzyme to be “reset” by the hydrolysis of the acyl-enzyme intermediate.

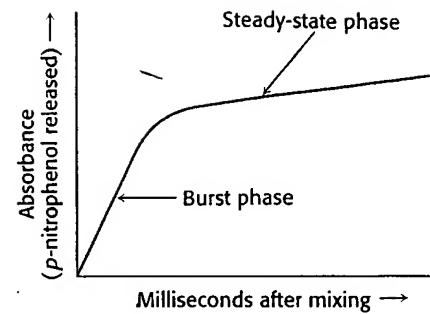


FIGURE 9.4 Kinetics of chymotrypsin catalysis. Two stages are evident in the cleaving of *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester by chymotrypsin: a rapid burst phase (pre-steady state) and a steady-state phase.

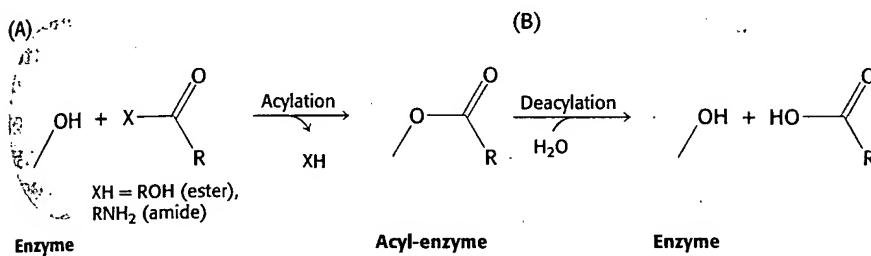


FIGURE 9.5 Covalent catalysis. Hydrolysis by chymotrypsin takes place in two stages: (A) acylation to form the acyl-enzyme intermediate followed by (B) deacylation to regenerate the free enzyme.

9.1.3 Serine is Part of a Catalytic Triad That Also Includes Histidine and Aspartic Acid



STRUCTURAL INSIGHTS, Chymotrypsin: A Serine Protease. Work with interactive molecular models to learn more about the structural bases of active site specificity and reactivity, and some of the ways in which active site residues can be identified.

The determination of the three-dimensional structure of chymotrypsin by David Blow in 1967 was a source of further insight into its mechanism of action. Overall, chymotrypsin is roughly spherical and comprises three polypeptide chains, linked by disulfide bonds. It is synthesized as a single polypeptide, termed chymotrypsinogen, which is activated by the proteolytic cleavage of the polypeptide to yield the three chains. The active site of chymotrypsin, marked by serine 195, lies in a cleft on the surface of the enzyme (Figure 9.6). The structural analysis revealed the chemical basis of the special reactivity of serine 195 (Figure 9.7). The side chain of serine 195 is hydrogen bonded to the imidazole ring of histidine 57. The $-\text{NH}$ group of this imidazole ring is, in turn, hydrogen

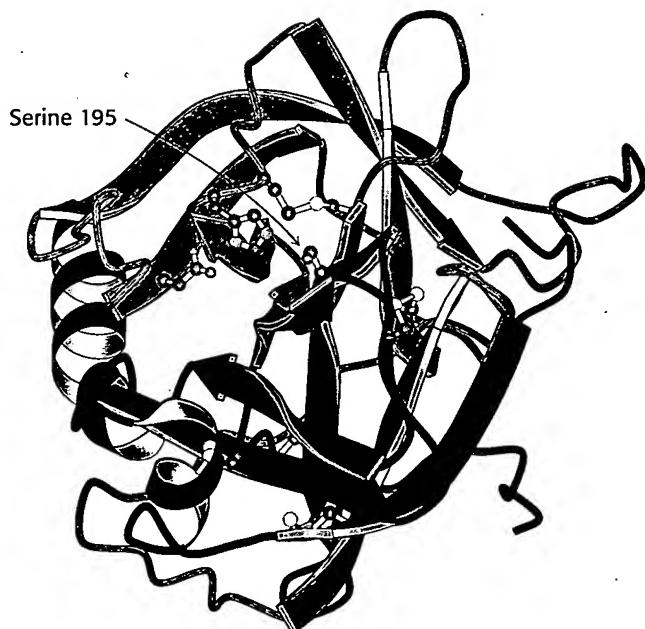


FIGURE 9.6 Three-dimensional structure of chymotrypsin. The three chains are shown in ribbon form in orange, blue, and green. The side chains of the catalytic triad residues, including serine 195, are shown as ball-and-stick representations, as are two intrastrand and interstrand disulfide bonds.

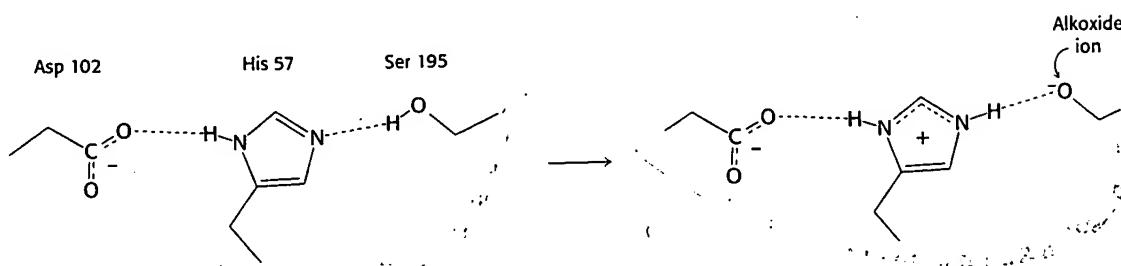
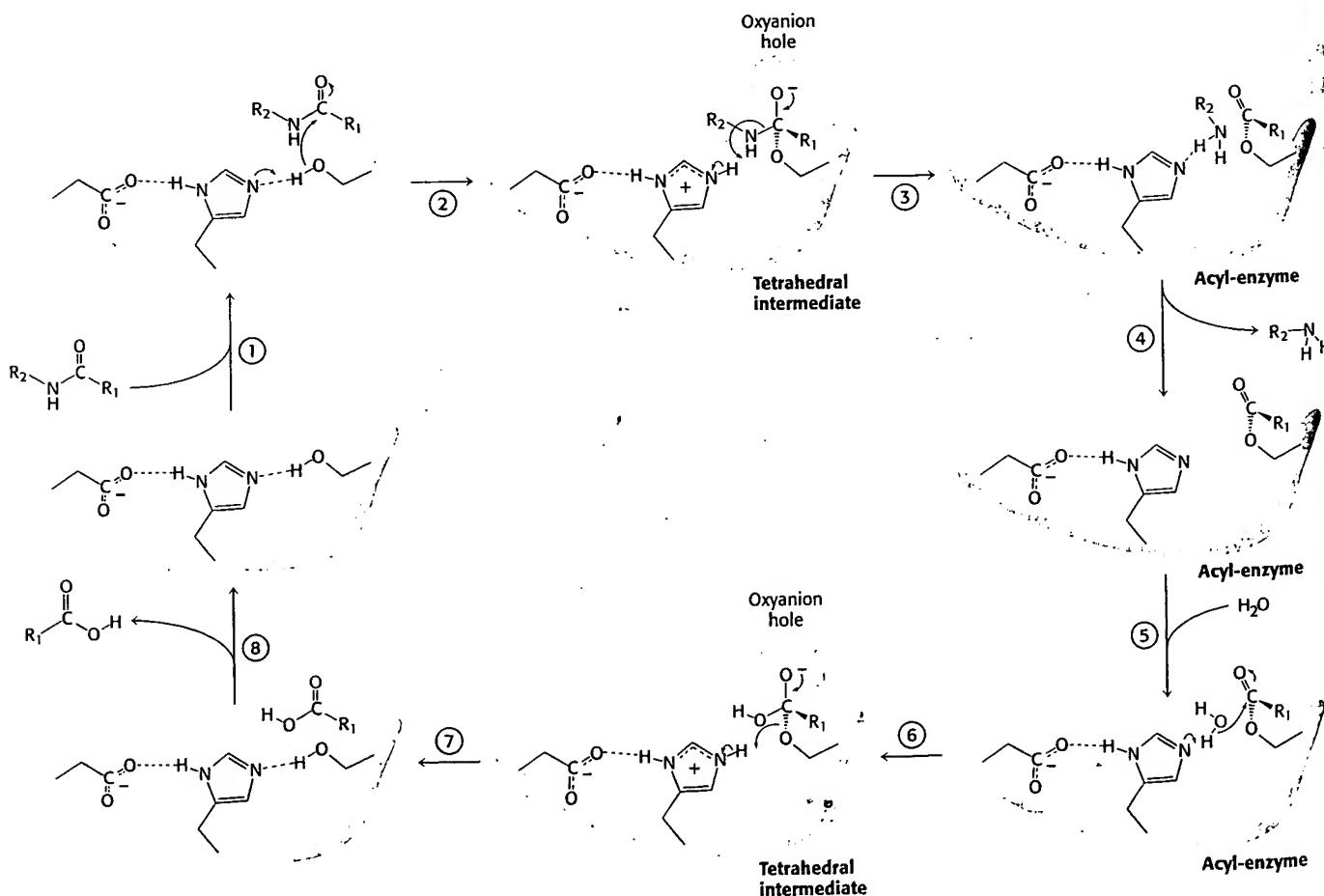


FIGURE 9.7 The catalytic triad. The catalytic triad, shown on the left, converts serine 195 into a potent nucleophile, as illustrated on the right.

FIGURE 9.8 Peptide hydrolysis by chymotrypsin. The mechanism of peptide hydrolysis illustrates the principles of covalent and acid-base catalysis. The dashed green lines indicate favorable interactions between the negatively charged aspartate residue and the positively charged histidine residue, which make the histidine residue a more powerful base.

bonded to the carboxylate group of aspartate 102. This constellation of residues is referred to as the *catalytic triad*. How does this arrangement of residues lead to the high reactivity of serine 195? The histidine residue serves to position the serine side chain and to polarize its hydroxyl group. In doing so, the residue acts as a general base catalyst, a hydrogen ion acceptor, because the polarized hydroxyl group of the serine residue is poised for deprotonation. The withdrawal of the proton from the hydroxyl group generates an alkoxide ion, which is a much more powerful nucleophile than an alcohol is. The aspartate residue helps orient the histidine residue and make it a better proton acceptor through electrostatic effects.

These observations suggest a mechanism for peptide hydrolysis (Figure 9.8). After substrate binding (step 1), the reaction begins with the hydroxyl group of serine 195 making a nucleophilic attack on the carbonyl carbon atom of the substrate (step 2): The nucleophilic attack changes the geometry around this carbon atom from trigonal planar to tetrahedral. The inherently unstable tetrahedral intermediate formed bears a formal negative



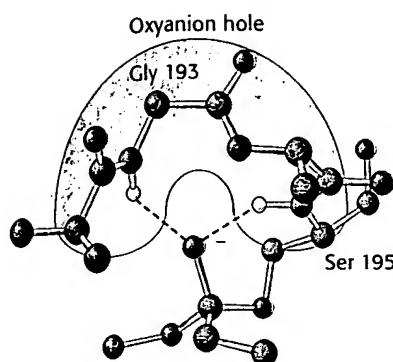


FIGURE 9.9 The oxyanion hole. The structure stabilizes the tetrahedral intermediate of the chymotrypsin reaction. Hydrogen bonds (shown in green) link peptide NH groups and the negatively charged oxygen atom.

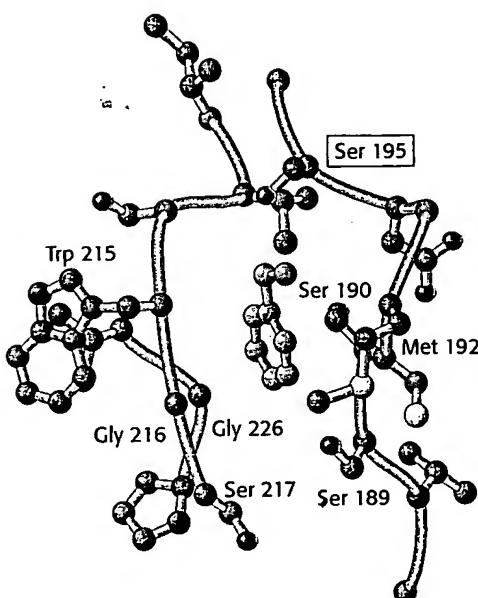


FIGURE 9.10 The hydrophobic pocket of chymotrypsin. The hydrophobic pocket of chymotrypsin is responsible for its substrate specificity. The key amino acids that constitute the binding site are labeled, including the active-site serine residue (boxed). The position of an aromatic ring bound in the pocket is shown in green.

charge on the oxygen atom derived from the carbonyl group. This charge is stabilized by interactions with NH groups from the protein in a site termed the *oxyanion hole* (Figure 9.9). These interactions also help stabilize the transition state that precedes the formation of the tetrahedral intermediate. This tetrahedral intermediate then collapses to generate the acyl-enzyme (step 3). This step is facilitated by the transfer of a proton from the positively charged histidine residue to the amino group formed by cleavage of the peptide bond. The amine component is now free to depart from the enzyme (step 4) and is replaced by a water molecule (step 5). The ester group of the acyl-enzyme is now hydrolyzed by a process that is essentially a repeat of steps 2 through 4. The water molecule attacks the carbonyl group while a proton is concomitantly removed by the histidine residue, which now acts as a general acid catalyst, forming a tetrahedral intermediate (step 6). This structure breaks down to form the carboxylic acid product (step 7). Finally, the release of the carboxylic acid product (step 8) readies the enzyme for another round of catalysis.

This mechanism accounts for all characteristics of chymotrypsin action except the observed preference for cleaving the peptide bonds just past residues with large, hydrophobic side chains. Examination of the three-dimensional structure of chymotrypsin with substrate analogs and enzyme inhibitors revealed the presence of a deep, relatively hydrophobic pocket, called the S_1 pocket, into which the long, uncharged side chains of residues such as phenylalanine and tryptophan can fit. *The binding of an appropriate side chain into this pocket positions the adjacent peptide bond into the active site for cleavage* (Figure 9.10). The specificity of chymotrypsin depends almost entirely on which amino acid is directly on the amino-terminal side of the peptide bond to be cleaved. Other proteases have more-complex specificity patterns, as illustrated in Figure 9.11. Such enzymes have

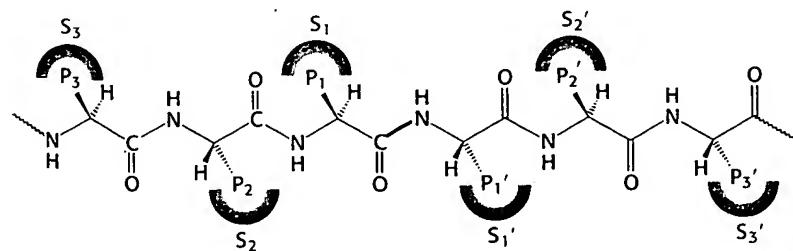


FIGURE 9.11 Specificity nomenclature for protease-substrate interactions. The potential sites of interaction of the substrate with the enzyme are designated P (shown in red), and corresponding binding sites on the enzyme are designated S. The scissile bond (also shown in red) is the reference point.

additional pockets on their surfaces for the recognition of other residues in the substrate. Residues on the amino-terminal side of the scissile bond (the bond to be cleaved) are labeled P_1 , P_2 , P_3 , and so forth, indicating their positions in relation to the scissile bond. Likewise, residues on the carboxyl side of the scissile bond are labeled P'_1 , P'_2 , P'_3 , and so forth. The corresponding sites on the enzyme are referred to as S_1 , S_2 or S'_1 , S'_2 , and so forth.

9.1.4 Catalytic Triads Are Found in Other Hydrolytic Enzymes

 Many other proteins have subsequently been found to contain catalytic triads similar to that discovered in chymotrypsin. Some, such as trypsin and elastase, are obvious homologs of chymotrypsin. The sequences of these proteins are approximately 40% identical with that of chymotrypsin, and their overall structures are nearly the same (Figure 9.12). These proteins operate by mechanisms identical with that of chymotrypsin. However, they have very different substrate specificities. Trypsin cleaves at the peptide bond after residues with long, positively charged side chains—namely, arginine and lysine—whereas elastase cleaves at the peptide bond after amino acids with small side chains—such as alanine and serine. Comparison of the S_1 pockets of these enzymes reveals the basis of the specificity. In trypsin, an aspartate residue (Asp 189) is present at the bottom of the S_1 pocket in place of a serine residue in chymotrypsin. The aspartate residue attracts and stabilizes a positively charged arginine or lysine residue in the substrate. In elastase, two residues at the top of the pocket in chymotrypsin and trypsin are replaced with valine (Val 190 and Val 216). These residues close off the mouth of the pocket so that only small side chains may enter (Figure 9.13).

Other members of the chymotrypsin family include a collection of proteins that take part in blood clotting, to be discussed in Chapter 10. In addition, a wide range of proteases found in bacteria and viruses also belong to this clan. Furthermore, other enzymes that are not homologs of chymotrypsin have been found to contain very similar active sites. As noted in Chapter 7, the presence of very similar active sites in these different protein families is a consequence of convergent evolution. Subtilisin, a protease in bacteria such as *Bacillus amyloliquefaciens*, is a particularly well characterized example. The active site of this enzyme includes both the catalytic triad and the oxyanion hole. However, one of the NH groups that forms the oxyanion hole comes from the side chain of an asparagine residue rather than from the peptide backbone (Figure 9.14). Subtilisin is the founding member of another large family of proteases that includes representatives from Archaea, Eubacteria, and Eukarya.

Yet another example of the catalytic triad has been found in carboxypeptidase II from wheat. The structure of this enzyme is not significantly similar to either chymotrypsin or subtilisin (Figure 9.15). This protein is a member of an intriguing family of homologous proteins that includes esterases such as acetylcholine esterase and certain lipases. These enzymes all make use of histidine-activated nucleophiles, but the nucleophiles may be cysteine rather than serine. Finally, other proteases have been discovered that contain an active-site serine or threonine residue that is activated not by a histidine-aspartate pair but by a primary amino group from the side chain of lysine or by the N-terminal amino group of the polypeptide chain.

Thus, the catalytic triad in proteases has emerged at least three times in the course of evolution. We can conclude that this catalytic strategy must be an especially effective approach to the hydrolysis of peptides and related bonds.

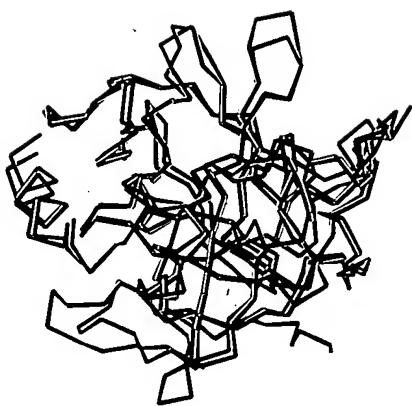


FIGURE 9.12 Structural similarity of trypsin and chymotrypsin.
An overlay of the structure of chymotrypsin (red) on that of trypsin (blue) shows the high degree of similarity. Only α -carbon atom positions are shown. The mean deviation in position between corresponding α -carbon atoms is 1.7 Å.

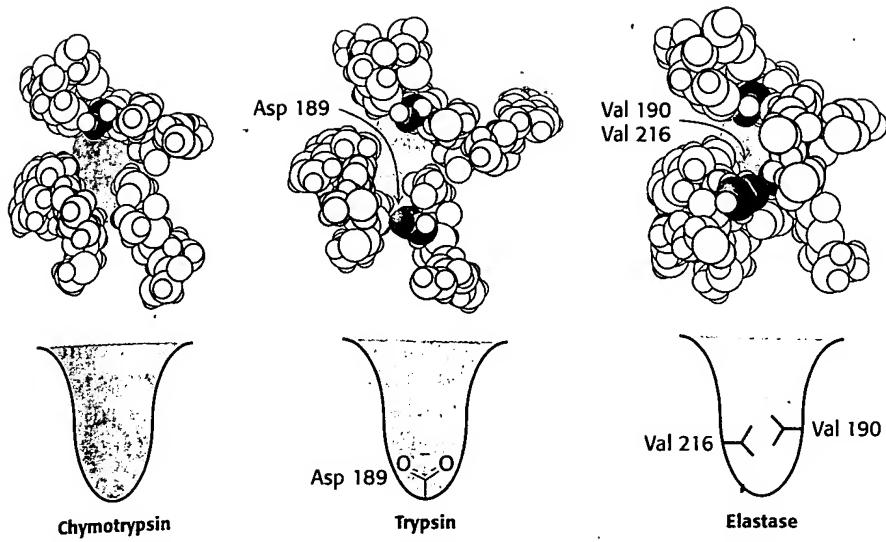


FIGURE 9.13 The S₁ pockets of chymotrypsin, trypsin, and elastase. Certain residues play key roles in determining the specificity of these enzymes. The side chains of these residues, as well as those of the active-site serine residues, are shown in color.

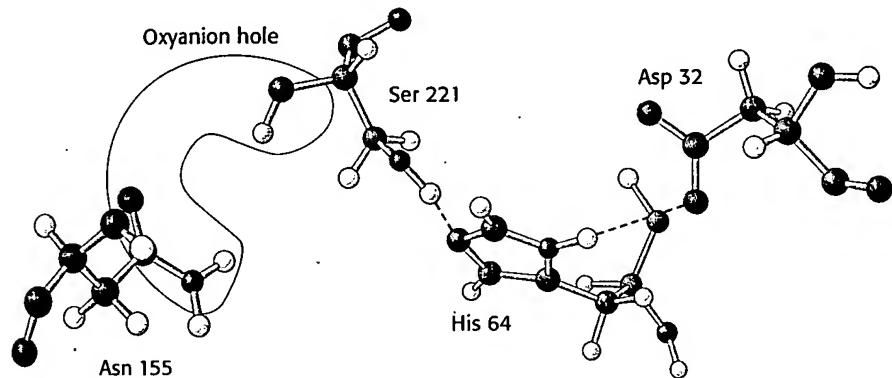


FIGURE 9.14 The catalytic triad and oxyanion hole of subtilisin. The peptide bond attacked by nucleophilic serine 221 of the catalytic triad will develop a negative charge, which is stabilized by enzyme NH groups (both in the backbone and in the side chain of Asn 155) located in the oxyanion hole.

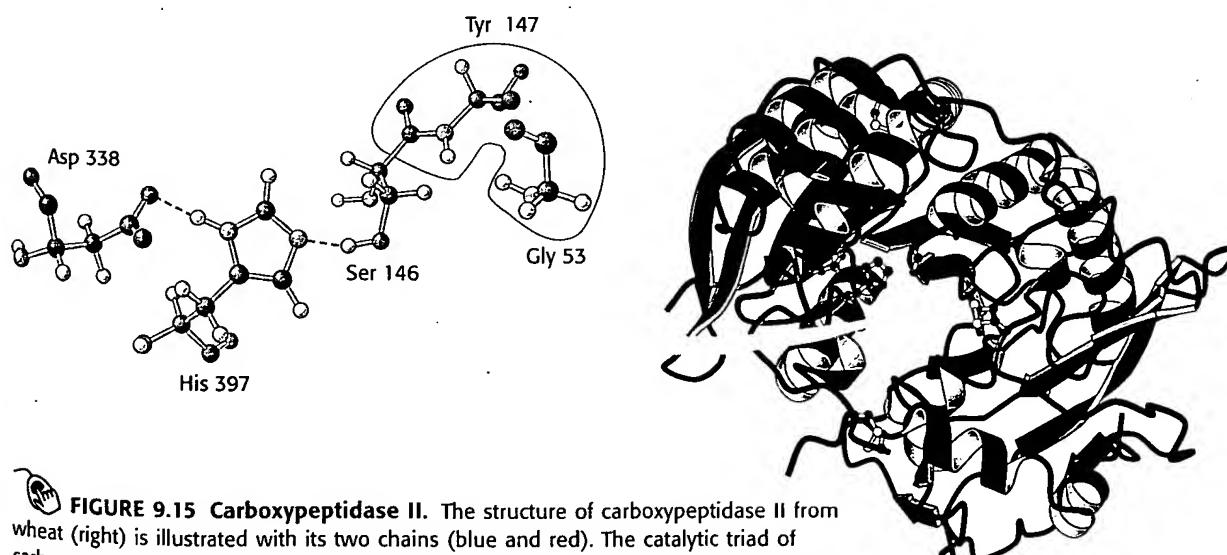


FIGURE 9.15 Carboxypeptidase II. The structure of carboxypeptidase II from wheat (right) is illustrated with its two chains (blue and red). The catalytic triad of carboxypeptidase II (left) is composed of the same amino acids as those in chymotrypsin, despite the fact that the enzymes display no structural similarity. The residues that form the oxyanion hole are highlighted in yellow.

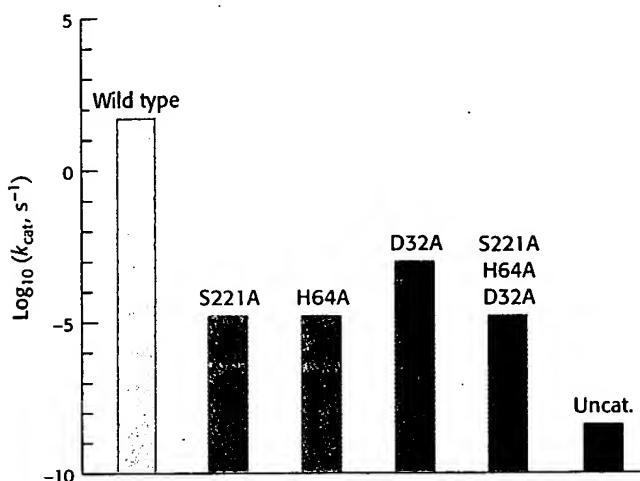


FIGURE 9.16 Site-directed mutagenesis of subtilisin. Residues of the catalytic triad were mutated to alanine, and the activity of the mutated enzyme was measured. Mutations in any component of the catalytic triad cause a dramatic loss of enzyme activity. Note that the activity is displayed on a logarithmic scale. The mutations are identified as follows: the first letter is the one-letter abbreviation for the amino acid being altered; the number identifies the position of the residue in the primary structure; and the second letter is the one-letter abbreviation for the amino acid replacing the original one.

9.1.5 The Catalytic Triad Has Been Dissected by Site-Directed Mutagenesis

The techniques of molecular biology discussed in Chapter 6 have permitted detailed examination of the catalytic triad. In particular, site-directed mutagenesis has been used to test the contribution of individual amino acid residues to the catalytic power of an enzyme. Subtilisin has been extensively studied by this method. Each of the residues within the catalytic triad, consisting of aspartic acid 32, histidine 64, and serine 221, has been individually converted into alanine, and the ability of each mutant enzyme to cleave a model substrate has been examined (Figure 9.16). As expected, the conversion of active-site serine 221 into alanine dramatically reduced catalytic power; the value of k_{cat} fell to less than *one-millionth* of its value for the wild-type enzyme.

The value of K_M was essentially unchanged: its increase by no more than a factor of two indicated that substrate binding is not significantly affected. The mutation of histidine 64 to alanine had very similar effects. These observations support the notion that the serine–histidine pair act together to generate a nucleophile of sufficient power to attack the carbonyl group of a peptide bond. The conversion of aspartate 32 into alanine had a smaller effect, although the value of k_{cat} still fell to less than 0.005% of its wild-type value. The simultaneously conversion of all three catalytic triad residues into alanine was no more deleterious than the conversion of serine or histidine alone. Despite the reduction in their catalytic power, the mutated enzymes still hydrolyze peptides a thousand times as rapidly as does buffer at pH 8.6.

Because the oxyanion hole of subtilisin includes a side-chain NH group in addition to backbone NH groups, it is possible to probe the importance of the oxyanion hole for catalysis by site-directed mutagenesis. The mutation of asparagine 155 to glycine reduced the value of k_{cat} to 0.2% of its wild-type value but increased the value of K_M by only a factor of two. These observations demonstrate that the NH group of the asparagine residue plays a significant role in stabilizing the tetrahedral intermediate and the transition state leading to it.

9.1.6 Cysteine, Aspartyl, and Metalloproteases Are Other Major Classes of Peptide-Cleaving Enzymes

Not all proteases utilize strategies based on activated serine residues. Classes of proteins have been discovered that employ three alternative approaches to peptide-bond hydrolysis (Figure 9.17). These classes are the (1) cysteine proteases, (2) aspartyl proteases, and (3) metalloproteases. In each case, the strategy generates a nucleophile that attacks the peptide carbonyl group (Figure 9.18).

The strategy used by the *cysteine proteases* is most similar to that used by the chymotrypsin family. In these enzymes, a cysteine residue, activated by a histidine residue, plays the role of the nucleophile that attacks the peptide bond (see Figure 9.18), in a manner quite analogous to that of the serine residue in serine proteases. An ideal example of these proteins is papain, an enzyme purified from the fruit of the papaya. Mammalian proteases homologous to papain have been discovered, most notably the cathepsins, proteins having a role in the immune and other systems. The cysteine-based active site arose independently at least twice in the course of evolution; the caspases, enzymes that play a major role in apoptosis (Section 2.4.3), have active sites similar to that of papain, but their overall structures are unrelated.

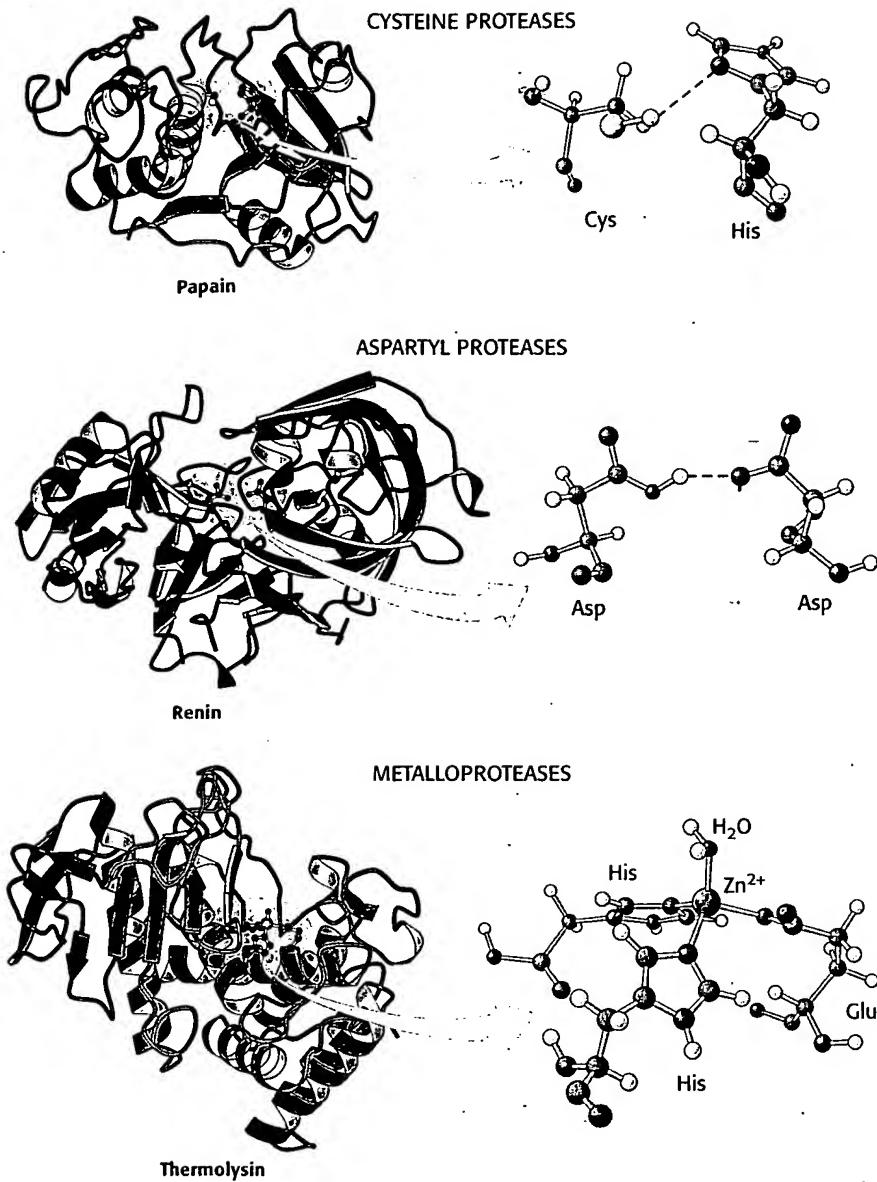
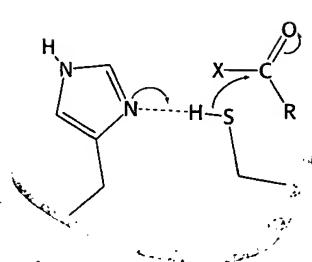
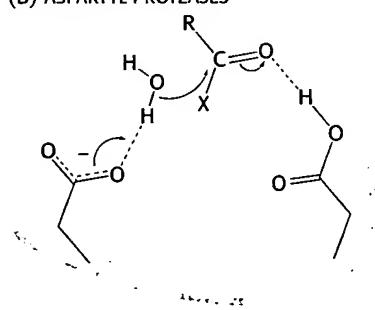


FIGURE 9.17 Three classes of proteases and their active sites. These examples of a cysteine protease, an aspartyl protease, and a metalloprotease use a histidine-activated cysteine residue, an aspartate-activated water molecule, and a metal-activated water molecule, respectively, as the nucleophile. The two halves of renin are in blue and red to highlight the approximate twofold symmetry of aspartyl proteases.

(A) CYSTEINE PROTEASES



(B) ASPARTYL PROTEASES



(C) METALLOPROTEASES

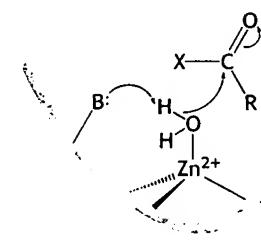


FIGURE 9.18 The activation strategies for three classes of proteases. The peptide carbonyl group is attacked by (A) a histidine-activated cysteine, in the cysteine proteases; (B) an aspartate-activated water molecule, in the aspartyl proteases; and (C) a metal-activated water molecule, in the metalloproteases. For the metalloproteases, the letter B represents a base (often a glutamate) that helps deprotonate the metal-bound water.

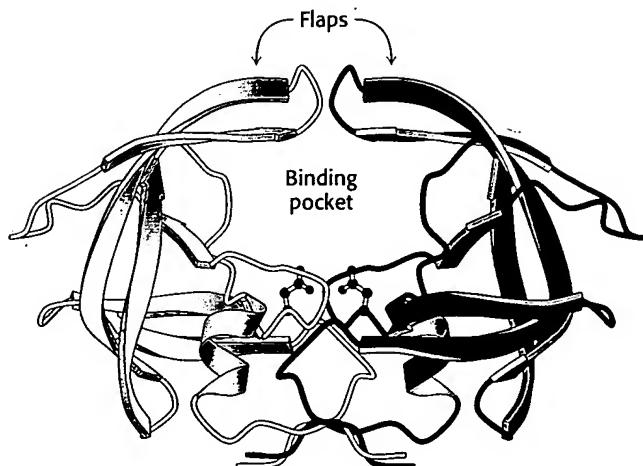
The second class comprises the *aspartyl proteases*. The central feature of the active sites is a pair of aspartic acid residues that act together to allow a water molecule to attack the peptide bond. One aspartic acid residue (in its deprotonated form) activates the attacking water molecule by poising it for deprotonation, whereas the other aspartic acid residue (in its protonated form) polarizes the peptide carbonyl, increasing its susceptibility to attack (see Figure 9.18). Members of this class include renin, an enzyme having a role in the regulation of blood pressure, and the digestive enzyme pepsin. These proteins possess approximate twofold symmetry, suggesting that the two halves are evolutionarily related. A likely scenario is that two copies of a gene for the ancestral enzyme fused to form a single gene that encoded a single-chain enzyme. Each copy of the gene would have contributed an aspartate residue to the active site. The human immunodeficiency virus (HIV) and other retroviruses contain an unfused dimeric aspartyl protease that is similar to the fused protein, but the individual chains are not joined to make a single chain (Figure 9.19). This observation is consistent with the idea that the enzyme may have originally existed as separate subunits.

The *metalloproteases* constitute the final major class of peptide-cleaving enzymes. The active site of such a protein contains a bound metal ion, almost always zinc, that activates a water molecule to act as a nucleophile to attack the peptide carbonyl group. The bacterial enzyme thermolysin and the digestive enzyme carboxypeptidase A are classic examples of the zinc proteases. Thermolysin, but not carboxypeptidase A, is a member of a large and diverse family of homologous zinc proteases that includes the matrix metalloproteases, enzymes that catalyze the reactions in tissue remodeling and degradation.

In each of these three classes of enzymes, the active site includes features that allow for the activation of water or another nucleophile as well as for the polarization of the peptide carbonyl group and subsequent stabilization of a tetrahedral intermediate (see Figure 9.18).

9.1.7 Protease Inhibitors Are Important Drugs

 **FIGURE 9.19 The structure of HIV protease and its binding pocket.** The protease is a dimer of identical subunits, shown in blue and yellow, consisting of 99 amino acids each. The active-site aspartic acid residues, one from each chain, are shown as ball-and-stick structures. The flaps will close down on the binding pocket after substrate has been bound.



 Compounds that block or modulate the activities of proteases can have dramatic biological effects. Most natural *protease inhibitors* are similar in structure to the peptide substrates of the enzyme that each inhibits (Section 10.5.4). Several important drugs are protease inhibitors. For example, captopril, an inhibitor of the metalloprotease angiotensin-converting enzyme (ACE), has been used to regulate blood pressure. Crixivan, an inhibitor of the HIV protease, is used in the treatment of AIDS. This protease cleaves multidomain viral proteins into their active forms; blocking this process completely prevents the virus from being infectious (see Figure 9.19). To prevent unwanted side effects, protease inhibitors used as drugs must be specific for one enzyme without inhibiting other proteins within the body.

Let us examine the interaction of Crixivan with HIV protease in more detail. Crixivan is constructed around an alcohol that mimics the tetrahedral intermediate; other groups are present to bind into the S_2 , S_1 , S_1' , and S_2' recognition sites on the enzyme (Figure 9.20). The results of x-ray crystallographic studies revealed the structure of the enzyme–Crixivan complex, showing that Crixivan adopts a conformation that approximates the twofold symmetry of the enzyme (Figure 9.21). The active site of HIV protease is covered by two apparently flexible flaps that fold down on top of the bound in-

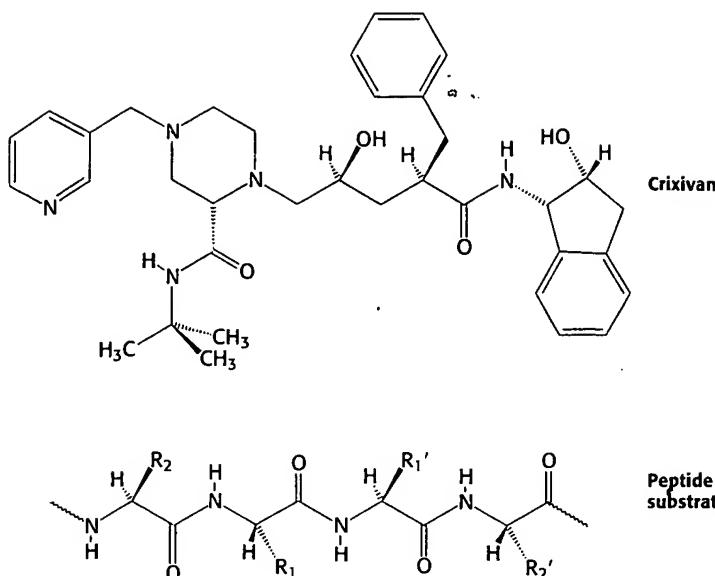


FIGURE 9.20 Crixivan, an HIV protease inhibitor. The structure of Crixivan is shown in comparison with that of a peptide substrate of HIV protease. The scissile bond in the substrate is highlighted in red.

hibitor. The hydroxyl group of the central alcohol interacts with two aspartate residues of the active site, one in each subunit. In addition, two carbonyl groups of the inhibitor are hydrogen bonded to a water molecule (not shown), which, in turn, is hydrogen bonded to a peptide NH group in each of the flaps. This interaction of the inhibitor with water and the enzyme is not possible with cellular aspartyl proteases such as renin and thus may contribute to the specificity of Crixivan and other inhibitors for HIV protease.

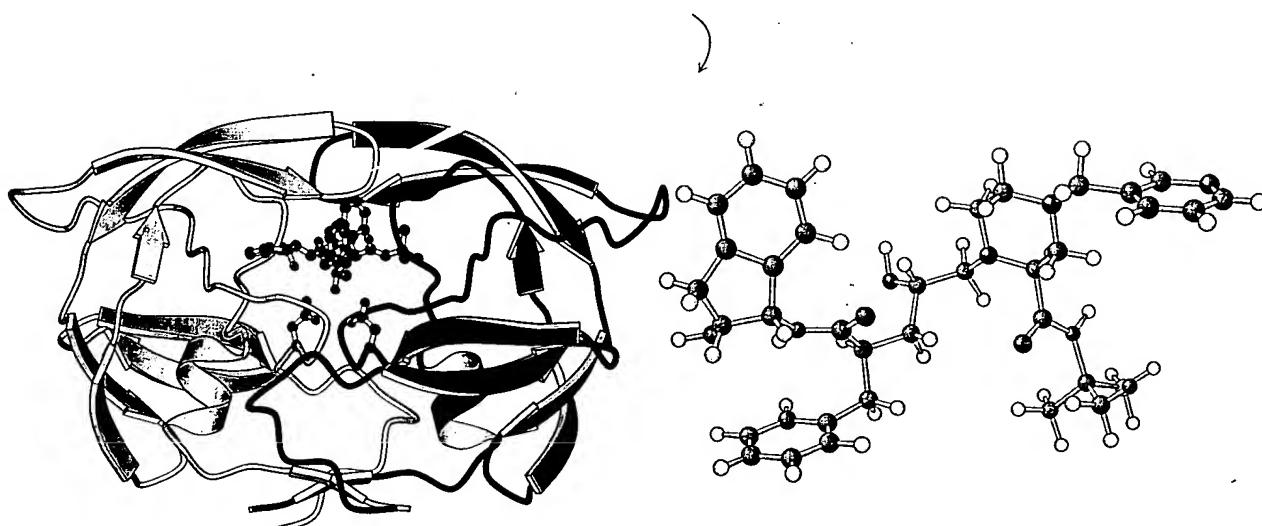
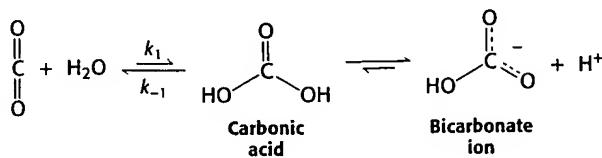


FIGURE 9.21 HIV protease–Crixivan complex. (Left) The HIV protease is shown with the inhibitor crixivan bound at the active site. (Right) The drug has been rotated to reveal its approximately twofold symmetric conformation.

9.2 MAKING A FAST REACTION FASTER: CARBONIC ANHYDRASES

Carbon dioxide is a major end product of aerobic metabolism. In complex organisms, this carbon dioxide is released into the blood and transported to the lungs for exhalation. While in the blood, carbon dioxide reacts with

water. The product of this reaction is a moderately strong acid, carbonic acid ($pK_a = 3.5$), which becomes bicarbonate ion on the loss of a proton.



Even in the absence of a catalyst, this hydration reaction proceeds at a moderate pace. At 37°C near neutral pH, the second-order rate constant k_1 is $0.0027 \text{ M}^{-1} \text{ s}^{-1}$. This corresponds to an effective first-order rate constant of 0.15 s^{-1} in water ($[\text{H}_2\text{O}] = 55.5 \text{ M}$). Similarly, the reverse reaction, the dehydration of bicarbonate, is relatively rapid, with a rate constant of $k_{-1} = 50 \text{ s}^{-1}$. These rate constants correspond to an equilibrium constant of $K_1 = 5.4 \times 10^{-5}$ and a ratio of $[\text{CO}_2]$ to $[\text{H}_2\text{CO}_3]$ of 340:1.



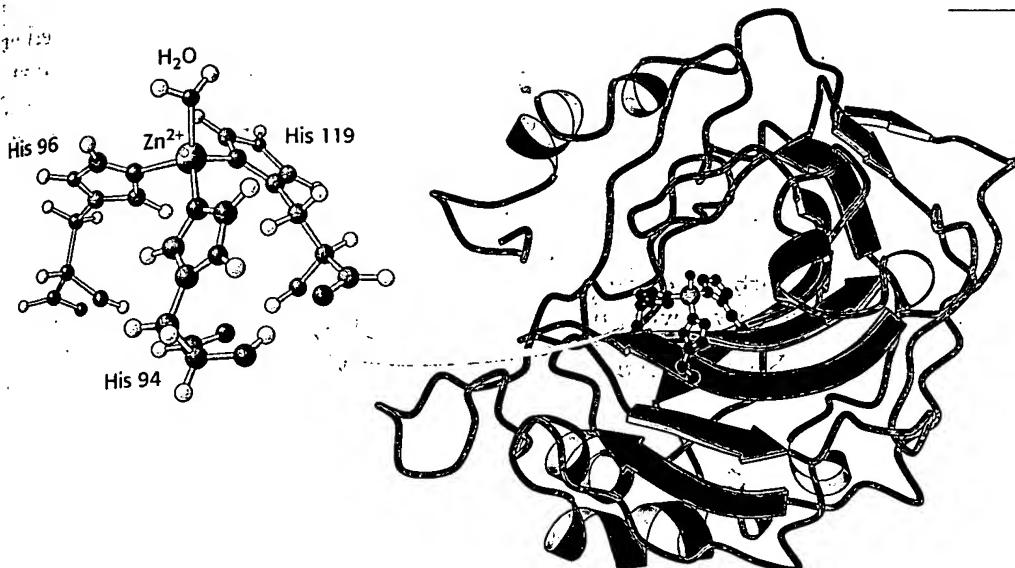
Despite the fact that CO_2 hydration and HCO_3^- dehydration occur spontaneously at reasonable rates in the absence of catalysts, almost all organisms contain enzymes, referred to as *carbonic anhydrases*, that catalyze these processes. Such enzymes are required because CO_2 hydration and HCO_3^- dehydration are often coupled to rapid processes, particularly transport processes. For example, HCO_3^- in the blood must be dehydrated to form CO_2 for exhalation as the blood passes through the lungs. Conversely, CO_2 must be converted into HCO_3^- for the generation of the aqueous humor of the eye and other secretions. Furthermore, both CO_2 and HCO_3^- are substrates and products for a variety of enzymes, and the rapid interconversion of these species may be necessary to ensure appropriate substrate levels. So important are these enzymes in human beings that mutations in some carbonic anhydrases have been found to cause osteopetrosis (excessive formation of dense bones accompanied by anemia) and mental retardation.

Carbonic anhydrases accelerate CO_2 hydration dramatically. The most active enzymes, typified by human carbonic anhydrase II, hydrate CO_2 at rates as high as $k_{\text{cat}} = 10^6 \text{ s}^{-1}$, or a million times a second. Fundamental physical processes such as diffusion and proton transfer ordinarily limit the rate of hydration, and so special strategies are required to attain such prodigious rates.

9.2.1 Carbonic Anhydrase Contains a Bound Zinc Ion Essential for Catalytic Activity

Less than 10 years after the discovery of carbonic anhydrase in 1932, this enzyme was found to contain bound zinc, associated with catalytic activity. This discovery, remarkable at the time, made carbonic anhydrase the first known zinc-containing enzyme. At present, hundreds of enzymes are known to contain zinc. In fact, more than one-third of all enzymes either contain bound metal ions or require the addition of such ions for activity. The chemical reactivity of metal ions—associated with their positive charges, with their ability to form relatively strong yet kinetically labile bonds, and, in some cases, with their capacity to be stable in more than one oxidation state—explains why catalytic strategies that employ metal ions have been adopted throughout evolution.

The results of x-ray crystallographic studies have supplied the most detailed and direct information about the zinc site in carbonic anhydrase. At least seven carbonic anhydrases, each with its own gene, are present in human beings. They are all clearly homologous, as revealed by substantial levels of sequence identity. Carbonic anhydrase II, present in relatively



 **FIGURE 9.22 The structure of human carbonic anhydrase II and its zinc site.** (Left) The zinc is bound to the imidazole rings of three histidine residues as well as to a water molecule. (Right) The location of the zinc site in the enzyme.

high concentrations in red blood cells, has been the most extensively studied (Figure 9.22).

Zinc is found only in the + 2 state in biological systems; so we need consider only this oxidation level as we examine the mechanism of carbonic anhydrase. A zinc atom is essentially always bound to four or more ligands; in carbonic anhydrase, three coordination sites are occupied by the imidazole rings of three histidine residues and an additional coordination site is occupied by a water molecule (or hydroxide ion, depending on pH). Because all of the molecules occupying the coordination sites are neutral, the overall charge on the $Zn(His)_3$ unit remains + 2.

9.2.2 Catalysis Entails Zinc Activation of Water

How does this zinc complex facilitate carbon dioxide hydration? A major clue comes from the pH profile of enzymatically catalyzed carbon dioxide hydration (Figure 9.23). At pH 8, the reaction proceeds near its maximal rate. As the pH decreases, the rate of the reaction drops. The midpoint of this transition is near pH 7, suggesting that a group with $pK_a = 7$ plays an important role in the activity of carbonic anhydrase and that the deprotonated (high pH) form of this group participates more effectively in catalysis. Although some amino acids, notably histidine, have pK_a values near 7, a variety of evidence suggests that the group responsible for this transition is not an amino acid but is the zinc-bound water molecule. Thus, the binding of a water molecule to the positively charged zinc center reduces the pK_a of the water molecule from 15.7 to 7 (Figure 9.24). With the lowered pK_a , a

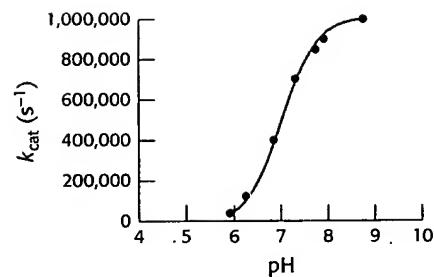


FIGURE 9.23 Effect of pH on carbonic anhydrase activity. Changes in pH alter the rate of carbon dioxide hydration catalyzed by carbonic anhydrase II. The enzyme is maximally active at high pH.

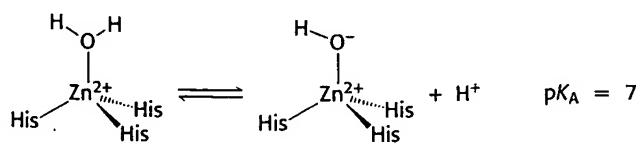


FIGURE 9.24 The pK_a of water-bound zinc. Binding to zinc lowers the pK_a of water from 15.7 to 7.

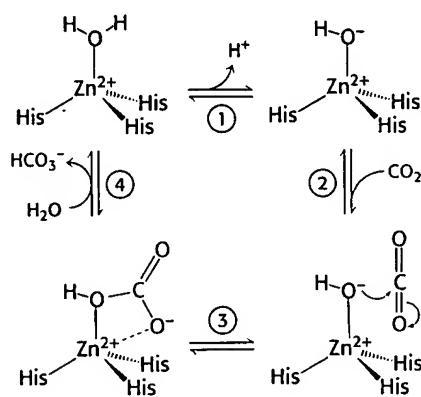


FIGURE 9.25 Mechanism of carbonic anhydrase. The zinc-bound hydroxide mechanism for the hydration of carbon dioxide catalyzed by carbonic anhydrase.

substantial concentration of hydroxide ion (bound to zinc) is generated at neutral pH. A zinc-bound hydroxide ion is sufficiently nucleophilic to attack carbon dioxide much more readily than water does. The importance of the zinc-bound hydroxide ion suggests a simple mechanism for carbon dioxide hydration (Figure 9.25).

1. Zinc facilitates the release of a proton from a water molecule, which generates a hydroxide ion.
2. The carbon dioxide substrate binds to the enzyme's active site and is positioned to react with the hydroxide ion.
3. The hydroxide ion attacks the carbon dioxide, converting it into bicarbonate ion.
4. The catalytic site is regenerated with the release of the bicarbonate ion and the binding of another molecule of water.

Thus, the binding of water to zinc favors the formation of the transition state, leading to bicarbonate formation by facilitating proton release and by bringing the two reactants into close proximity. A range of studies supports this mechanism. In particular, studies of a *synthetic analog model system* provide evidence for its plausibility. A simple synthetic ligand binds zinc through four nitrogen atoms (compared with three histidine nitrogen atoms in the enzyme), as shown in Figure 9.26. One water molecule remains bound to the zinc ion in the complex. Direct measurements reveal that this water molecule has a pK_a value of 8.7, not as low as the value for the water molecule in carbonic anhydrase but substantially lower than the value for free water. At pH 9.2, this complex accelerates the hydration of carbon dioxide more than 100-fold. Although catalysis by this synthetic system is much less efficient than catalysis by carbonic anhydrase, the model system strongly suggests that the zinc-bound hydroxide mechanism is likely to be correct. Carbonic anhydrases have evolved to utilize the reactivity intrinsic to a zinc-bound hydroxide ion as a potent catalyst.

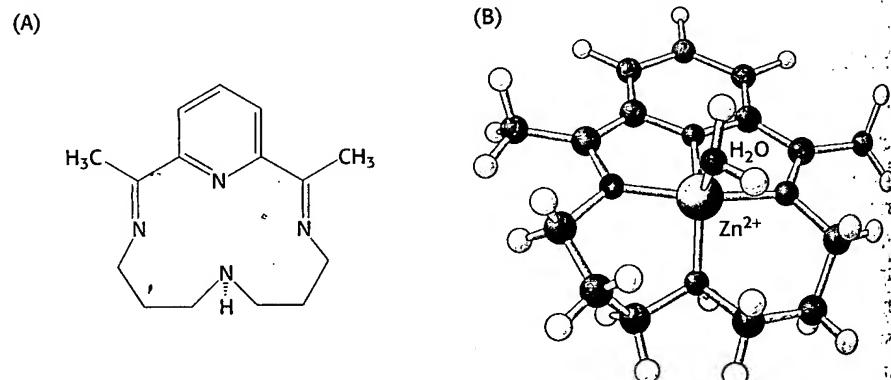


FIGURE 9.26 A synthetic analog model system for carbonic anhydrase. (A) An organic compound, capable of binding zinc, was synthesized as a model for carbonic anhydrase. The zinc complex of this ligand accelerates the hydration of carbon dioxide more than 100-fold under appropriate conditions. (B) The structure of the presumed active complex showing zinc bound to the ligand and to one water molecule.

9.2.3 A Proton Shuttle Facilitates Rapid Regeneration of the Active Form of the Enzyme

As noted earlier, some carbonic anhydrases can hydrate carbon dioxide at rates as high as a million times a second (10^6 s^{-1}). The magnitude of this rate can be understood from the following observations. At the conclusion of a carbon dioxide hydration reaction, the zinc-bound water molecule must lose a proton to regenerate the active form of the enzyme (Figure 9.27). The rate of the reverse reaction, the protonation of the zinc-bound hydroxide ion, is limited by the rate of proton diffusion. Protons diffuse very rapidly with second-

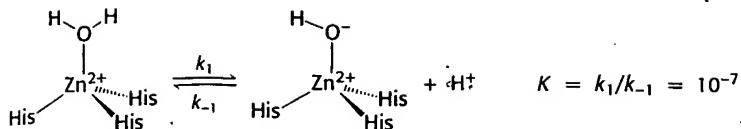


FIGURE 9.27 Kinetics of water deprotonation. The kinetics of deprotonation and protonation of the zinc-bound water molecule in carbonic anhydrase.

order rate constants near $10^{-11} \text{ M}^{-1} \text{ s}^{-1}$. Thus, the backward rate constant k_{-1} must be less than $10^{11} \text{ M}^{-1} \text{ s}^{-1}$. Because the equilibrium constant K is equal to k_1/k_{-1} , the forward rate constant is given by $k_1 = K \cdot k_{-1}$. Thus, if $k_{-1} \leq 10^{11} \text{ M}^{-1} \text{ s}^{-1}$ and $K = 10^{-7} \text{ M}$ (because $\text{p}K_a = 7$), then k_1 must be less than or equal to 10^4 s^{-1} . In other words, the rate of proton diffusion limits the rate of proton release to less than 10^4 s^{-1} for a group with $\text{p}K_a = 7$. However, if carbon dioxide is hydrated at a rate of 10^6 s^{-1} , then every step in the mechanism (see Figure 9.25) must take place at least this fast. How can this apparent paradox be resolved?

The answer became clear with the realization that *the highest rates of carbon dioxide hydration require the presence of buffer, suggesting that the buffer components participate in the reaction*. The buffer can bind or release protons. The advantage is that, whereas the concentrations of protons and hydroxide ions are limited to 10^{-7} M at neutral pH, the concentration of buffer components can be much higher, on the order of several millimolar. If the buffer component BH^+ has a $\text{p}K_a$ of 7 (matching that for the zinc-bound water), then the equilibrium constant for the reaction in Figure 9.28 is 1.

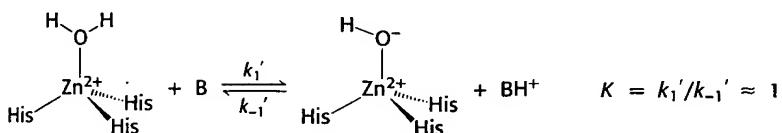
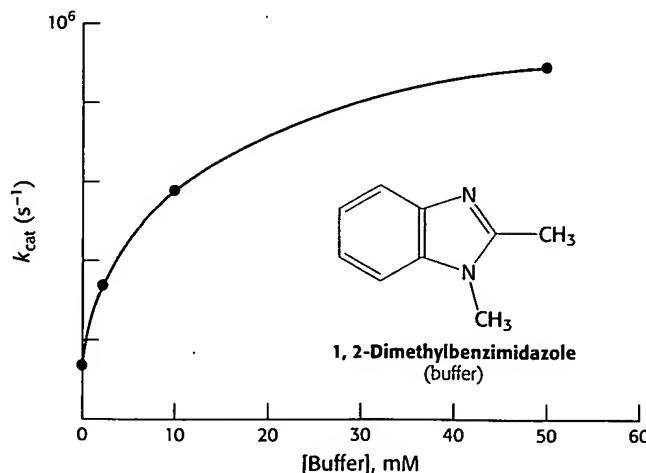


FIGURE 9.28 The effect of buffer on deprotonation. The deprotonation of the zinc-bound water molecule in carbonic anhydrase is aided by buffer component B.

The rate of proton abstraction is given by $k_1' \cdot [\text{B}]$. The second-order rate constants k_1' and k_{-1}' will be limited by buffer diffusion to values less than approximately $10^9 \text{ M}^{-1} \text{ s}^{-1}$. Thus, buffer concentrations greater than $[\text{B}] = 10^{-3} \text{ M}$ (1 mM) may be high enough to support carbon dioxide hydration rates of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ because $k_1' \cdot [\text{B}] = (10^9 \text{ M}^{-1} \text{ s}^{-1}) \cdot (10^{-3} \text{ M}) = 10^6 \text{ s}^{-1}$. This prediction is confirmed experimentally (Figure 9.29).

The molecular components of many buffers are too large to reach the active site of carbonic anhydrase. Carbonic anhydrase II has evolved a *proton shuttle* to allow buffer components to participate in the reaction from solution. The primary component of this shuttle is histidine 64. This residue transfers protons from the zinc-bound water molecule to the protein surface and then to the buffer (Figure 9.30). Thus, *catalytic function has been enhanced through the evolution of an apparatus for controlling proton transfer from and to the active site*. Because protons participate in many biochemical reactions, the manipulation of the proton inventory within active sites is crucial to the function of many enzymes and explains the prominence of acid-base catalysis.

FIGURE 9.29 The effect of buffer concentration on the rate of carbon dioxide hydration. The rate of carbon dioxide hydration increases with the concentration of the buffer 1,2-dimethylbenzimidazole. The buffer enables the enzyme to achieve its high catalytic rates.



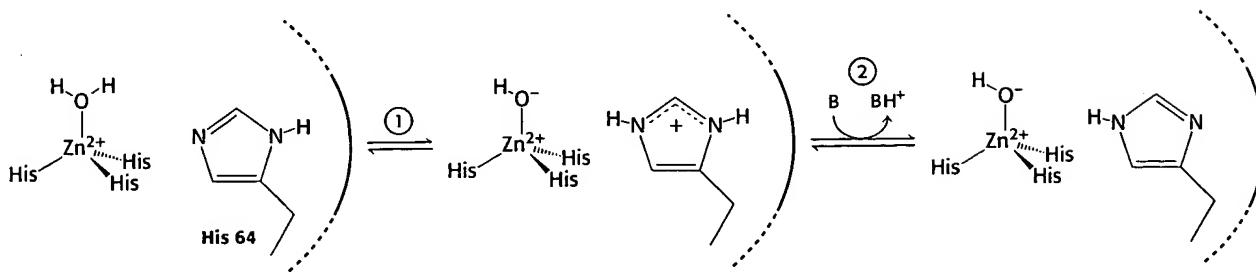
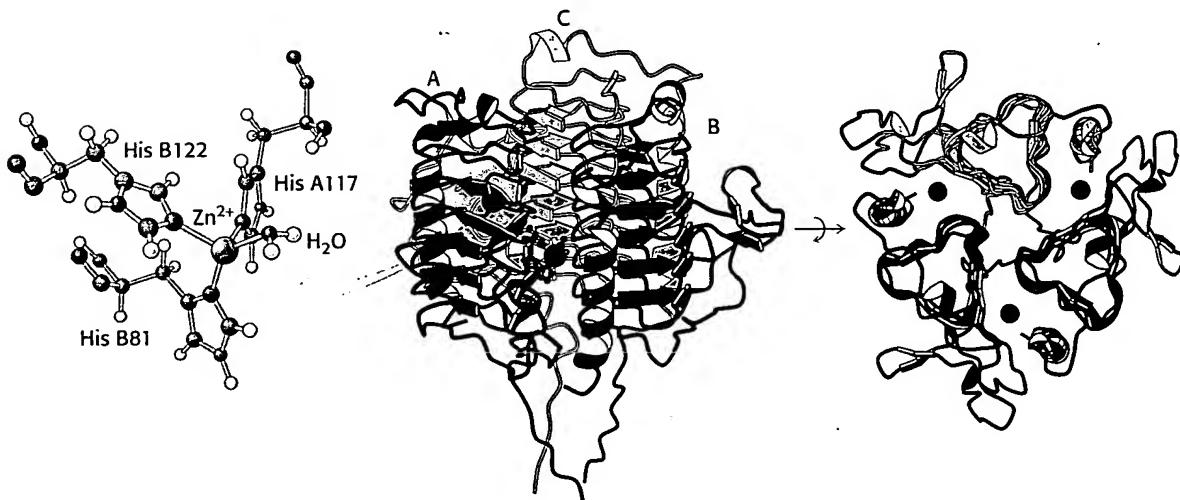


FIGURE 9.30 Histidine proton shuttle.

(1) Histidine 64 abstracts a proton from the zinc bound water molecule, generating a nucleophilic hydroxide ion and a protonated histidine. (2) The buffer (B) removes a proton from the histidine, regenerating the unprotonated form.

9.2.4 Convergent Evolution Has Generated Zinc-Based Active Sites in Different Carbonic Anhydrases

 Carbonic anhydrases homologous to the human enzymes, referred to as α -carbonic anhydrases, are common in animals and in some bacteria and algae. In addition, two other families of carbonic anhydrases have been discovered. The β -carbonic anhydrases are found in higher plants and in many bacterial species, including *E. coli*. These proteins contain the zinc required for catalytic activity but are not significantly similar in sequence to the α -carbonic anhydrases. Furthermore, the β -carbonic anhydrases have only one conserved histidine residue, whereas the α -carbonic anhydrases have three. No three-dimensional structure is yet available, but spectroscopic studies suggest that the zinc is bound by one histidine residue, two cysteine residues (conserved among β -carbonic anhydrases), and a water molecule. A third family, the γ -carbonic anhydrases, also has been identified, initially in the archaeon *Methanoscincina thermophila*. The crystal structure of this enzyme reveals three zinc sites extremely similar to those in the α -carbonic anhydrases. In this case, however, the three zinc sites lie at the interfaces between the three subunits of a trimeric enzyme (Figure 9.31). The very striking left-handed β -helix (a β strand twisted into a left-handed helix) structure present in this enzyme has also been found in enzymes that catalyze reactions unrelated to those of carbonic anhydrase. Thus, convergent evolution has generated carbonic anhydrases that rely on coordinated zinc ions at least three times. In each



 **FIGURE 9.31 γ -Carbonic anhydrase.** (Left) The zinc site of γ -carbonic anhydrase. (Middle) The trimeric structure of the protein (individual chains are labeled A, B, and C). (Right) The protein is rotated to show a top-down view that highlights its threefold symmetry and the position of the zinc sites (green) at the interfaces between subunits.

case, the catalytic activity appears to be associated with zinc-bound water molecules.

9.3 RESTRICTION ENZYMES: PERFORMING HIGHLY SPECIFIC DNA-CLEAVAGE REACTIONS

Let us next consider a hydrolytic reaction that results in the cleavage of DNA. Bacteria and archaea have evolved mechanisms to protect themselves from viral infections. Many viruses inject their DNA genomes into cells; once inside, the viral DNA hijacks the cell's machinery to drive the production of viral proteins and, eventually, of progeny virus. Often, a viral infection results in the death of the host. A major protective strategy for the host is to use *restriction endonucleases* (restriction enzymes) to degrade the viral DNA on its introduction into a cell. These enzymes recognize particular base sequences, called *recognition sequences* or *recognition sites*, in their target DNA and cleave that DNA at defined positions. The most well studied class of restriction enzymes comprises the so-called type II restriction enzymes, which cleave DNA *within* their recognition sequences. Other types of restriction enzymes cleave DNA at positions somewhat distant from their recognition sites.

Restriction endonucleases must show tremendous specificity at two levels. First, they must cleave only DNA molecules that contain recognition sites (hereafter referred to as *cognate DNA*) without cleaving DNA molecules that lack these sites. Suppose that a recognition sequence is six base pairs long. Because there are 4^6 , or 4096, sequences having six base pairs, the concentration of sites that must not be cleaved will be approximately 5000-fold as high as the concentration of sites that should be cleaved. Thus, to keep from damaging host-cell DNA, endonucleases must cleave cognate DNA molecules much more than 5000 times as efficiently as they cleave nonspecific sites. Second, restriction enzymes must not degrade the host DNA. How do these enzymes manage to degrade viral DNA while sparing their own?

The restriction endonuclease *EcoRV* (from *E. coli*) cleaves double-stranded viral DNA molecules that contain the sequence 5'-GATATC-3' but leaves intact host DNA containing hundreds of such sequences. The host DNA is protected by other enzymes called methylases, which methylate adenine bases within host recognition sequences (Figure 9.32). For each restriction endonuclease, the host cell produces a corresponding methylase that marks the host DNA and prevents its degradation. These pairs of enzymes are referred to as *restriction-modification systems*. We shall return to the mechanism used to achieve the necessary levels of specificity after considering the chemistry of the cleavage process.

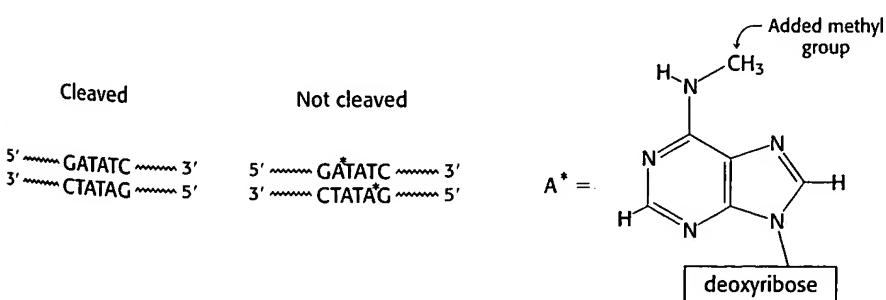
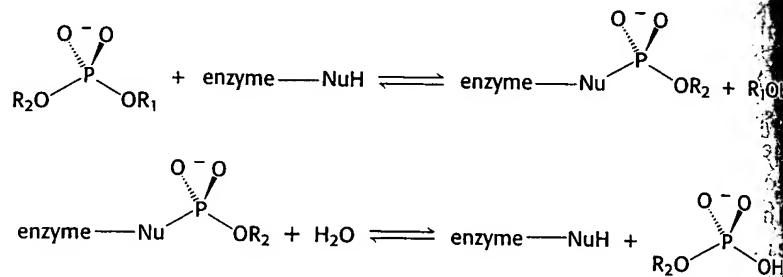


FIGURE 9.32 Protection by methylation. The recognition sequence for *EcoRV* endonuclease (left) and the sites of methylation (right) in DNA protected from the catalytic action of the enzyme.

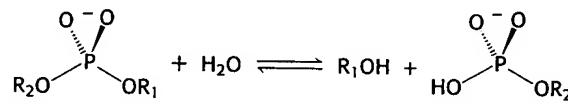
9.3.1 Cleavage Is by In-Line Displacement of 3' Oxygen from Phosphorus by Magnesium-Activated Water

The fundamental reaction catalyzed by restriction endonucleases is hydrolysis of the phosphodiester backbone of DNA. Specifically, the bond between the 3' oxygen atom and the phosphorus atom is broken. The products of this reaction are DNA strands with a free 3'-hydroxyl group and a 5'-phosphoryl group (Figure 9.33). This reaction proceeds by nucleophilic attack at the phosphorus atom. We will consider two types of mechanisms, as suggested by analogy with the proteases. The restriction endonucleases might cleave DNA in mechanism 1 through a covalent intermediate, employing a potent nucleophile (NuH), or in mechanism 2 by direct hydrolysis.

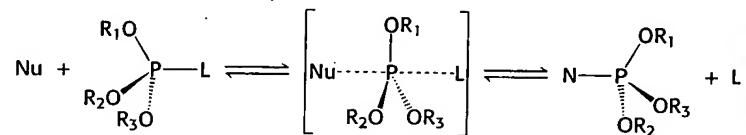
Mechanism Type 1 (covalent intermediate)



Mechanism Type 2 (direct hydrolysis)



Each postulates a different nucleophile to carry out the attack on the phosphorus. In either case, each reaction takes place by an *in-line displacement* path:



The incoming nucleophile attacks the phosphorus atom, and a pentacoordinate transition state is formed. This species has a trigonal bipyramidal geometry centered at the phosphorus atom, with the incoming nucleophile at one

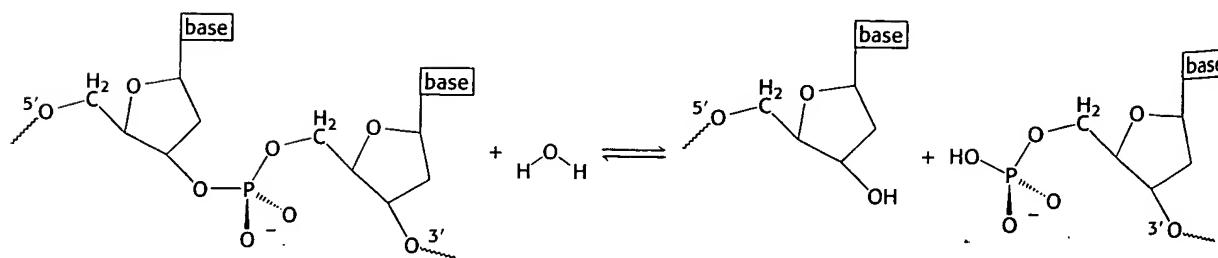


FIGURE 9.33 Hydrolysis of a phosphodiester bond. All restriction enzymes catalyze the hydrolysis of DNA phosphodiester bonds, leaving a phosphoryl group attached to the 5' end. The bond that is cleaved is shown in red.

apex of the two pyramids and the group that is displaced (the leaving group, L) at the other apex. The two mechanisms differ in the number of times the displacement occurs in the course of the reaction.

In the first type of mechanism, a nucleophile in the enzyme (analogous to serine 195 in chymotrypsin) attacks the phosphoryl group to form a covalent intermediate. In a second step, this intermediate is hydrolyzed to produce the final products. Because two displacement reactions take place at the phosphorus atom in the first mechanism, the stereochemical configuration at the phosphorus atom would be inverted and then inverted again, and the overall configuration would be retained. In the second type of mechanism, analogous to that used by the aspartyl and metalloproteases, an activated water molecule attacks the phosphorus atom directly. In this mechanism, a single displacement reaction takes place at the phosphorus atom. Hence, the stereochemical configuration of the tetrahedral phosphorus atom is inverted each time a displacement reaction takes place. Monitoring the stereochemical changes of the phosphorus could be one approach to determining the mechanism of restriction endonuclease action.

A difficulty is that the phosphorus centers in DNA are not chiral, because two of the groups bound to the phosphorus atom are simple oxygen atoms, identical with each other. This difficulty can be circumvented by preparing DNA molecules that contain chiral phosphoryl groups, made by replacing one oxygen atom with sulfur (called a phosphorothioate). Let us consider *EcoRV* endonuclease. This enzyme cleaves the phosphodiester bond between the T and the A at the center of the recognition sequence 5'-GATATC-3'. The first step in monitoring the activity of the enzyme is to synthesize an appropriate substrate for *EcoRV* containing phosphorothioates at the sites of cleavage (Figure 9.34). The reaction is then performed in water that has been greatly enriched in ^{18}O to allow the incoming oxygen atom to be marked. The location of the ^{18}O label with respect to the sulfur atom indicates whether the reaction proceeds with inversion or retention of stereochemistry. The analysis revealed that the stereochemical configuration at the phosphorus atom was inverted only once with cleavage. This result is consistent with a direct attack of water at phosphorus and rules out the formation of any covalently bound intermediate (Figure 9.35).

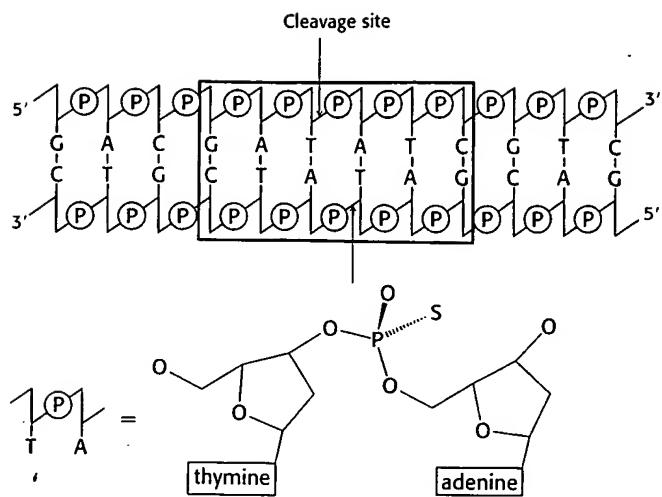


FIGURE 9.34 Labeling with phosphorothioates. Phosphorothioates, groups in which one of the nonbridging oxygen atoms is replaced with a sulfur atom, can be used to label specific sites in the DNA backbone to determine the overall stereochemical course of a displacement reaction. Here, a phosphorothioate is placed at sites that can be cleaved by *EcoRV* endonuclease.

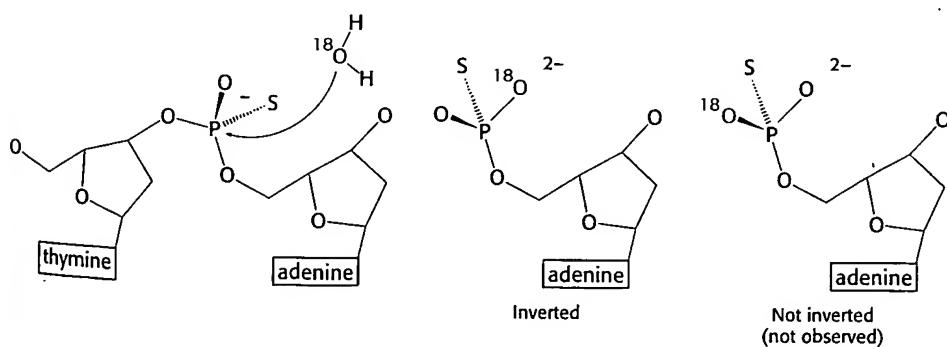


FIGURE 9.35 Stereochemistry of cleaved DNA. Cleavage of DNA by *EcoRV* endonuclease results in overall inversion of the stereochemical configuration at the phosphorus atom, as indicated by the stereochemistry of the phosphorus atom bound to one bridging oxygen atom, one ^{16}O , one ^{18}O , and one sulfur atom. This configuration strongly suggests that the hydrolysis takes place by the direct attack of water on the phosphorus atom.

9.3.2 Restriction Enzymes Require Magnesium for Catalytic Activity

Restriction endonucleases as well as many other enzymes that act on phosphate-containing substrates require Mg^{2+} or some other similar divalent cation for activity. What is the function of this metal?

It has been possible to examine the interactions of the magnesium ion when it is bound to the enzyme. Crystals have been produced of EcoRV endonuclease bound to oligonucleotides that contain the appropriate recognition sequences. These crystals are grown in the absence of magnesium to prevent cleavage; then, when produced, the crystals are soaked in solutions containing the metal. No cleavage takes place, allowing the location of the magnesium ion binding sites to be determined (Figure 9.36). The magnesium ion was found to be bound to six ligands: three are water molecules, two are carboxylates of the enzyme's aspartate residues, and one is an oxygen atom of the phosphoryl group at the site of cleavage. The magnesium ion holds a water molecule in a position from which the water molecule can attack the phosphoryl group and, in conjunction with the aspartate residues, helps polarize the water molecule toward deprotonation. Because cleavage does not take place within these crystals, the observed structure cannot be the true catalytic conformation. Additional studies have revealed that a second magnesium ion must be present in an adjacent site for EcoRV endonuclease to cleave its substrate.

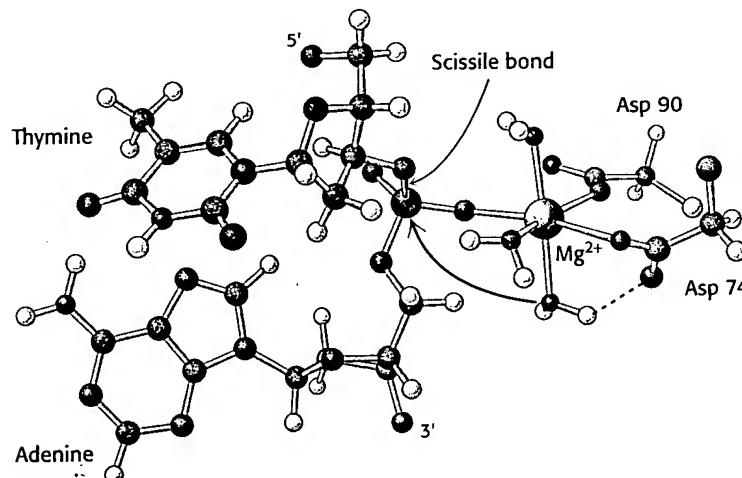


FIGURE 9.36 Magnesium ion binding site in EcoRV endonuclease. The magnesium ion helps to activate a water molecule and positions it so that it can attack the phosphate.

9.3.3 The Complete Catalytic Apparatus Is Assembled Only Within Complexes of Cognate DNA Molecules, Ensuring Specificity

We now return to the question of specificity, the defining feature of restriction enzymes. The recognition sequences for most restriction endonucleases are *inverted repeats*. This arrangement gives the three-dimensional structure of the recognition site a *twofold rotational symmetry* (Figure 9.37). The restriction enzymes display a corresponding symmetry to facilitate recognition: they are dimers whose two subunits are related by twofold rotational symmetry. The matching symmetry of the recognition sequence and the enzyme has been confirmed by the determination of the structure of the complex between EcoRV endonuclease and DNA fragments containing its recognition sequence (Figure 9.38). The enzyme surrounds the DNA in a tight embrace. Examination of this structure reveals features that are highly significant in determining specificity.

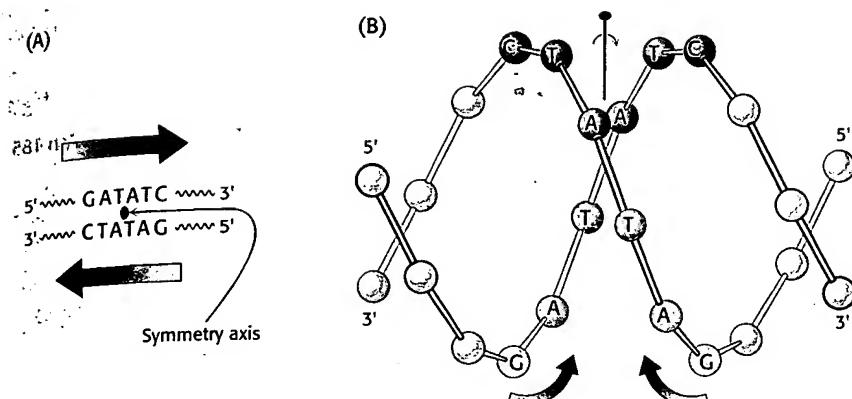


FIGURE 9.37 Structure of the recognition site of EcoRV endonuclease. (A) The sequence of the recognition site, which is symmetric around the axis of rotation designated in green. (B) The inverted repeat within the recognition sequence of EcoRV (and most other restriction endonucleases) endows the DNA site with twofold rotational symmetry.

A unique set of interactions occurs between the enzyme and a cognate DNA sequence. Within the 5'-GATATC-3' sequence, the G and A bases at the 5' end of each strand and their Watson-Crick partners directly contact the enzyme by hydrogen bonding with residues that are located in two loops, one projecting from the surface of each enzyme subunit (Figure 9.39). The most striking feature of this complex is the *distortion of the DNA*, which is substantially kinked in the center (Figure 9.40). The central two TA base pairs in the recognition sequence play a key role in producing the kink. They do not make contact with the enzyme but appear to be required because of their ease of distortion. 5'-TA-3' sequences are known to be among the most easily deformed base pairs. The distortion of the DNA at this site has severe effects on the specificity of enzyme action.

Specificity is often determined by an enzyme's binding affinity for substrates: In regard to EcoRV endonuclease, however, binding studies performed in the absence of magnesium have demonstrated that the enzyme binds to all sequences, both cognate and noncognate, with approximately equal affinity. However, the structures of complexes formed with noncognate DNA fragments are strikingly different from those formed with cognate DNA: the noncognate DNA conformation is not substantially distorted (Figure 9.41). *This lack of distortion has important consequences with regard to catalysis. No phosphate is positioned sufficiently close to the active-site*

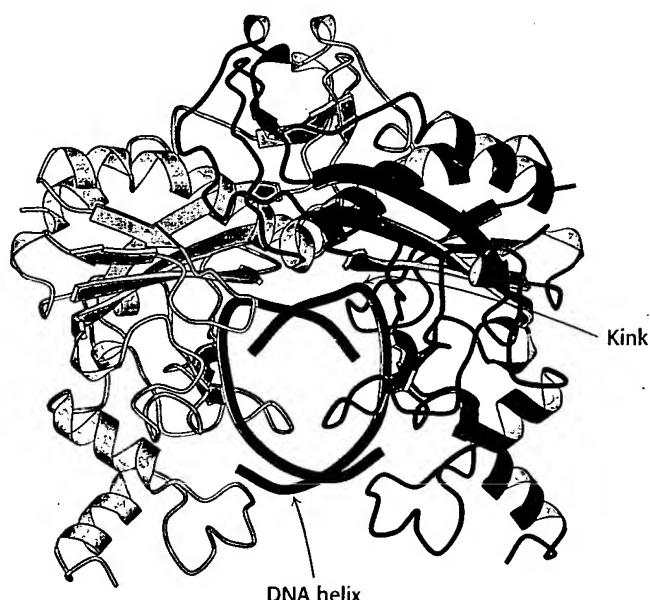
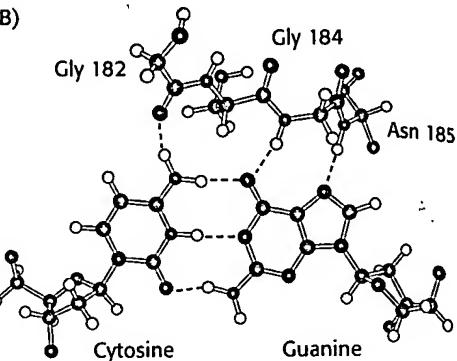


FIGURE 9.38 Structure of the EcoRV-cognate DNA complex. This view of the structure of EcoRV endonuclease bound to a cognate DNA fragment is down the helical axis of the DNA. The two protein subunits are in yellow and blue, and the DNA backbone is in red. The twofold axes of the enzyme dimer and the DNA are aligned.

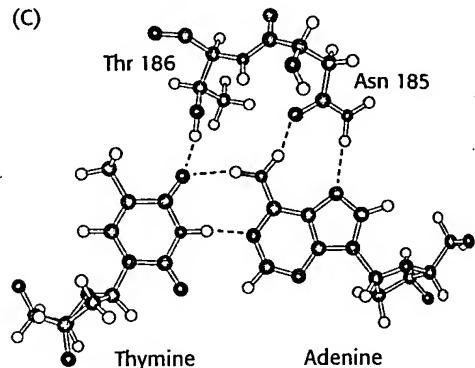
(A)



(B)



(C)

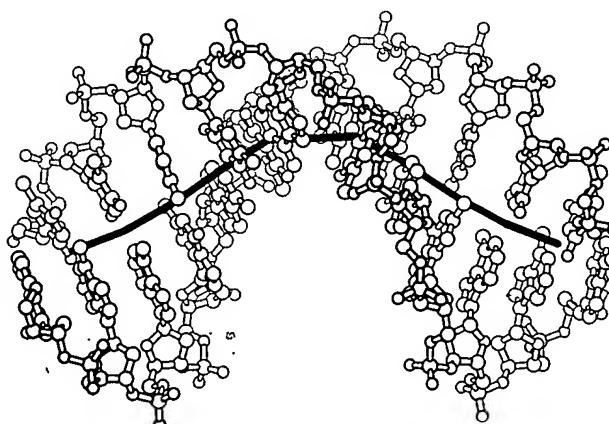


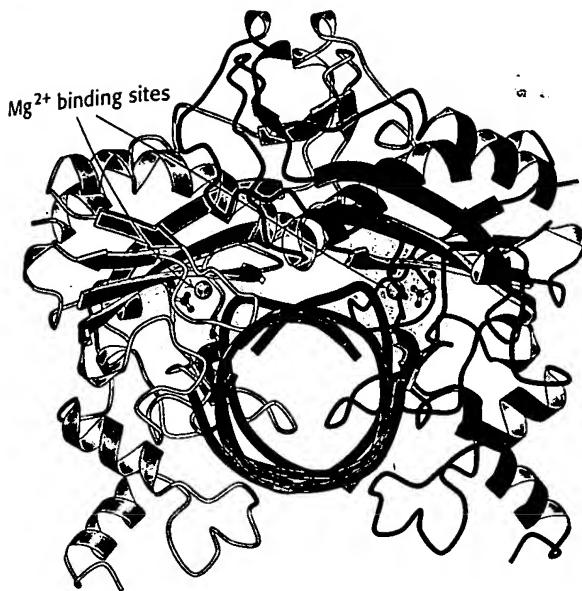
 **FIGURE 9.39 Hydrogen bonding interactions between EcoRV endonuclease and its DNA substrate.** One of the DNA-binding loops (in green) of EcoRV endonuclease is shown interacting with the base pairs of its cognate DNA binding site. Key amino acid residues are shown hydrogen bonding with (B) a CG base pair and (C) an AT base pair.

aspartate residues to complete a magnesium ion binding site (see Figure 9.36). Hence, the nonspecific complexes do not bind the magnesium ion and the complete catalytic apparatus is never assembled. The distortion of the substrate and the subsequent binding of the magnesium ion account for the catalytic specificity of more than 1,000,000-fold that is observed for *EcoRV* endonuclease despite very little preference at the level of substrate binding.

We can now see the role of binding energy in this strategy for attaining catalytic specificity. In binding to the enzyme, the DNA is distorted in such a way that additional contacts are made between the enzyme and the substrate, increasing the binding energy. However, this increase is canceled by the energetic cost of distorting the DNA from its relaxed conformation (Figure 9.42). Thus, for *EcoRV* endonuclease, there is little difference in binding affinity for cognate and nonspecific DNA fragments. However, the distortion in the cognate complex dramatically affects catalysis by

FIGURE 9.40 Distortion of the recognition site. The DNA is represented as a ball-and-stick model. The path of the DNA helical axis, shown in red, is substantially distorted on binding to the enzyme. For the B form of DNA, the axis is straight (not shown).





 **FIGURE 9.41 Nonspecific and cognate DNA within EcoRV endonuclease.** A comparison of the positions of the nonspecific (orange) and the cognate DNA (red) within EcoRV reveals that, in the nonspecific complex, the DNA backbone is too far from the enzyme to complete the magnesium ion binding sites.

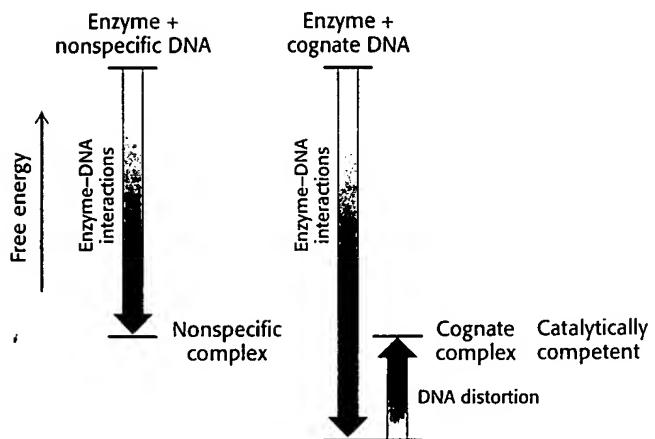


FIGURE 9.42 Greater binding energy of EcoRV endonuclease bound to cognate versus noncognate DNA. The additional interactions between EcoRV endonuclease and cognate DNA increase the binding energy, which can be used to drive DNA distortions necessary for forming a catalytically competent complex.

completing the magnesium ion binding site. *This example illustrates how enzymes can utilize available binding energy to deform substrates and poised them for chemical transformation.* Interactions that take place within the distorted substrate complex stabilize the transition state leading to DNA hydrolysis.

The distortion in the DNA explains how methylation blocks catalysis and protects host-cell DNA. When a methyl group is added to the amino group of the adenine nucleotide at the 5' end of the recognition sequence, the methyl group's presence precludes the formation of a hydrogen bond between the amino group and the side-chain carbonyl group of asparagine 185 (Figure 9.43). This asparagine residue is closely linked to the other amino acids that form specific contacts with the DNA. The absence of the hydrogen bond disrupts other interactions between the enzyme and the DNA substrate, and the distortion necessary for cleavage will not take place.

9.3.4 Type II Restriction Enzymes Have a Catalytic Core in Common and Are Probably Related by Horizontal Gene Transfer

 Type II restriction enzymes are prevalent in Archaea and Eubacteria. What can we tell of the evolutionary history of these enzymes? Comparison of the amino acid sequences of a variety of type II restriction endonucleases did not reveal significant sequence similarity between most pairs of enzymes. However, a careful examination of three-dimensional structures, taking into account the location of the active sites, revealed the presence of a core structure conserved in the different enzymes. This structure includes β strands that contain the aspartate (or, in some cases, glutamate) residues forming the magnesium ion binding sites (Figure 9.44).

These observations indicate that many type II restriction enzymes are indeed evolutionary related. Analyses of the sequences in greater detail suggest that bacteria may have obtained genes encoding these enzymes from

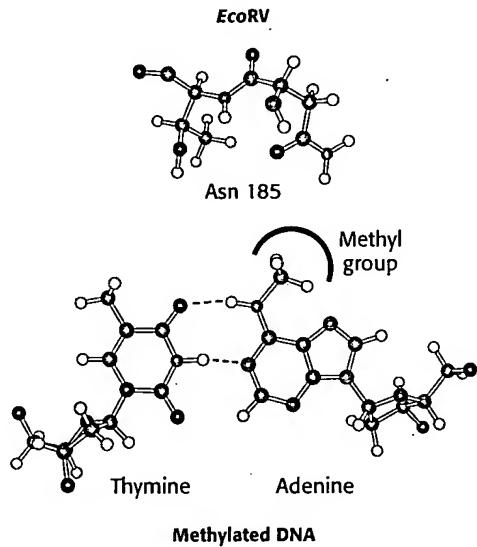


FIGURE 9.43 Methylation of adenine. The methylation of adenine blocks the formation of hydrogen bonds between EcoRV endonuclease and cognate DNA molecules and prevents their hydrolysis.

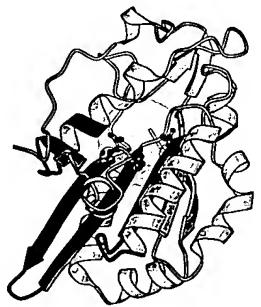
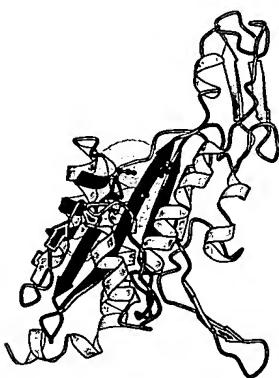


FIGURE 9.44 A conserved structural core in type II restriction enzymes. Four conserved structural elements, including the active-site region (in blue), are highlighted in color in these models of a single monomer from each dimeric enzyme. The positions of the amino acid sequences that form these elements within each overall sequence are represented schematically below each structure.

other species by *horizontal gene transfer*, the passing between species of pieces of DNA (such as plasmids) that provide a selective advantage in a particular environment. For example, *EcoRI* (from *E. coli*) and *RsrI* (from *Rhodobacter sphaeroides*) are 50% identical in sequence over 266 amino acids, clearly indicative of a close evolutionary relationship. However, these species of bacteria are not closely related, as is known from sequence comparisons of other genes and other evidence. Thus, *it appears that these species obtained the gene for this restriction endonuclease from a common source more recently than the time of their evolutionary divergence*. Moreover, the gene encoding *EcoRI* endonuclease uses particular codons to specify given amino acids that are strikingly different from the codons used by most *E. coli* genes, which suggests that the gene did not originate in *E. coli*. Horizontal gene transfer may be a relatively common event. For example, genes that inactivate antibiotics are often transferred, leading to the transmission of antibiotic resistance from one species to another. For restriction-modification systems, protection against viral infections may have favored horizontal gene transfer.

9.4 NUCLEOSIDE MONOPHOSPHATE KINASES: CATALYZING PHOSPHORYL GROUP EXCHANGE BETWEEN NUCLEOTIDES WITHOUT PROMOTING HYDROLYSIS

The final enzymes that we shall consider are the nucleoside monophosphate kinases (NMP kinases), typified by adenylate kinase. These enzymes catalyze the transfer of the terminal phosphoryl group from a nucleoside triphosphate (NTP), usually ATP, to the phosphoryl group on a nucleoside monophosphate (Figure 9.45). The challenge for NMP kinases is to promote the transfer of the phosphoryl group from NTP to NMP without promoting the competing reaction—the transfer of a phosphoryl group from NTP to water; that is, NTP hydrolysis. We shall see how the use of induced fit by these enzymes is used to solve this problem. Moreover, these enzymes employ metal ion catalysis; but, in this case, the metal forms a complex with the substrate to enhance enzyme–substrate interaction.

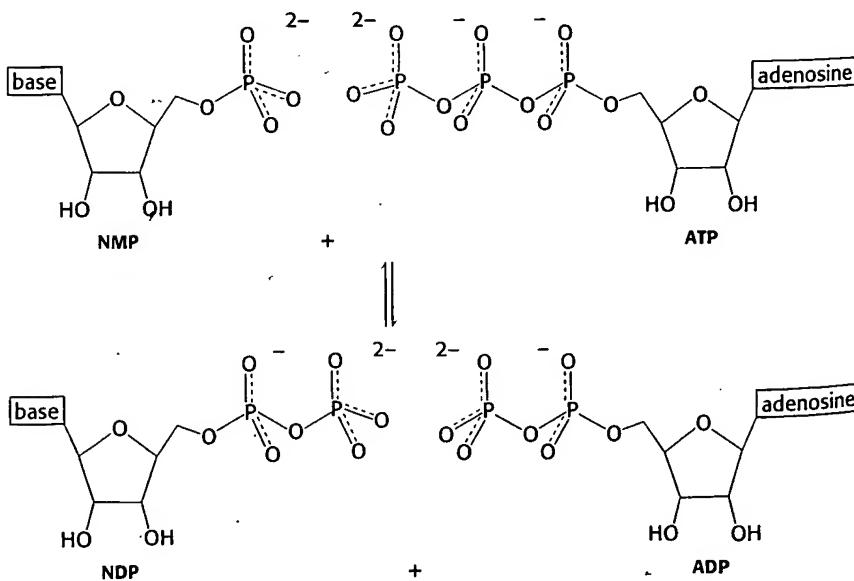


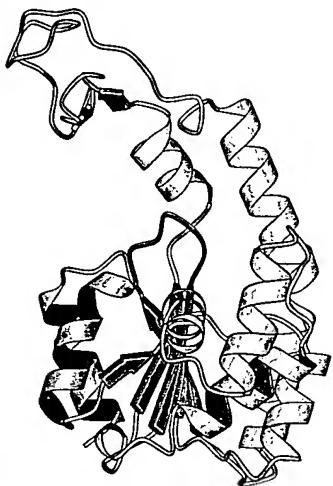
FIGURE 9.45 Phosphoryl group transfer by nucleoside monophosphate kinases. These enzymes catalyze the interconversion of nucleoside triphosphate (here, ATP) and a nucleoside monophosphate (NMP) into two nucleoside diphosphates by the transfer of a phosphoryl group (shown in red).

9.4.1 NMP Kinases Are a Family of Enzymes Containing P-Loop Structures

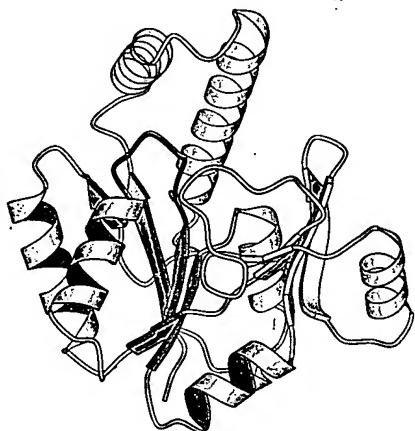
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NMP Kinases

X-ray crystallographic methods have yielded the three-dimensional structures of a number of different NMP kinases, both free and bound to substrates or substrate analogs. Comparison of these structures reveals that these enzymes form a family of homologous proteins (Figure 9.46). In particular, such comparisons reveal the presence of a conserved NTP-binding domain. This domain consists of a central β sheet, surrounded on both sides by α helices (Figure 9.47). A characteristic feature of this domain is a loop between the first β strand and the first helix. This loop, which typically has an amino acid sequence of the form Gly-X-X-X-X-Gly-Lys, is often referred to as the *P-loop* because it interacts with phosphoryl groups on the bound nucleotide (Figure 9.48). As described in Section 9.4.4, similar domains containing P-loops are present in a wide variety of important nucleotide-binding proteins.



Adenylate kinase



Guanylate kinase



FIGURE 9.47 The core domain of NMP kinases. The P-loop is shown in green. The dashed lines represent the remainder of the protein structure.

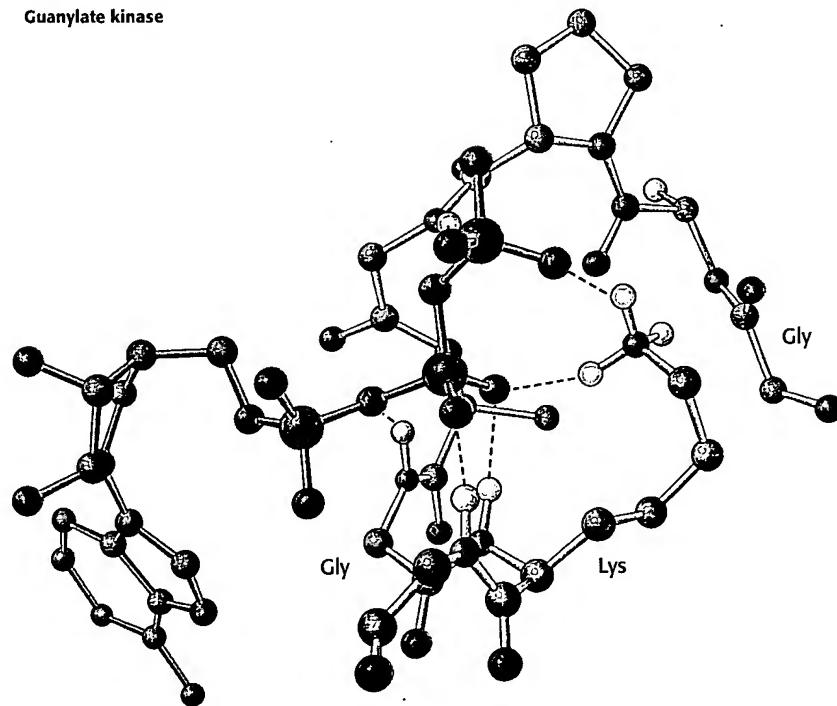


FIGURE 9.48 P-loop interaction with ATP. The P-loop of adenylate kinase interacts with the phosphoryl groups of ATP (shown with dark bonds). Hydrogen bonds (green) link ATP to peptide NH groups as well as a lysine residue conserved among NMP kinases.



FIGURE 9.46 Structures of adenylate kinase and guanylate kinase. The nucleoside triphosphate-binding domain is a common feature in these and other homologous nucleotide kinases. The domain consists of a central β -pleated sheet surrounded on both sides by α helices (highlighted in purple) as well as a key loop (shown in green).

9.4.2 Magnesium (or Manganese) Complexes of Nucleoside Triphosphates Are the True Substrates for Essentially All NTP-Dependent Enzymes

Kinetic studies of NMP kinases, as well as many other enzymes having ATP or other nucleoside triphosphates as a substrate, reveal that these enzymes are essentially inactive in the absence of divalent metal ions such as magnesium (Mg^{2+}) or manganese (Mn^{2+}), but acquire activity on the addition of these ions. In contrast with the enzymes discussed so far, the metal is not a component of the active site. Rather, nucleotides such as ATP bind these ions, and it is the metal ion–nucleotide complex that is the true substrate for the enzymes. The dissociation constant for the ATP– Mg^{2+} complex is approximately 0.1 mM, and thus, given that intracellular Mg^{2+} concentrations are typically in the millimolar range, essentially all nucleoside triphosphates are present as NTP– Mg^{2+} complexes.

How does the binding of the magnesium ion to the nucleotide affect catalysis? There are a number of related consequences, but all serve to enhance the specificity of the enzyme–substrate interactions by enhancing binding energy. First, the magnesium ion neutralizes some of the negative charges present on the polyphosphate chain, reducing nonspecific ionic interactions between the enzyme and the polyphosphate group of the nucleotide. Second, the interactions between the magnesium ion and the oxygen atoms in the phosphoryl group hold the nucleotide in well-defined conformations that can be specifically bound by the enzyme (Figure 9.49). Magnesium ions are typically coordinated to six groups in an octahedral arrangement. Typically, two oxygen atoms are directly coordinated to a magnesium ion, with the remaining coordination positions often occupied

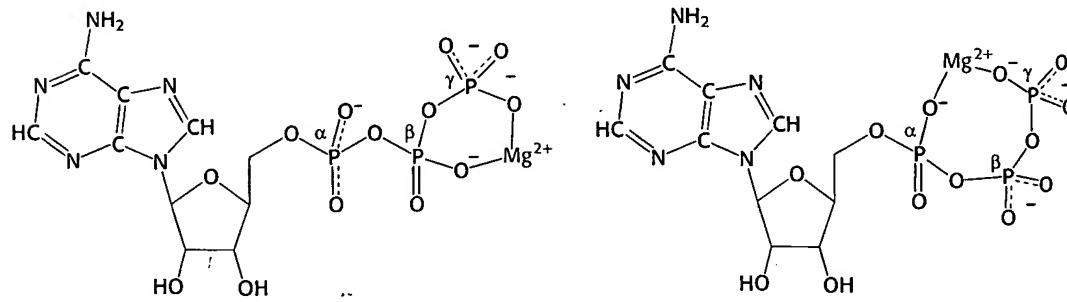
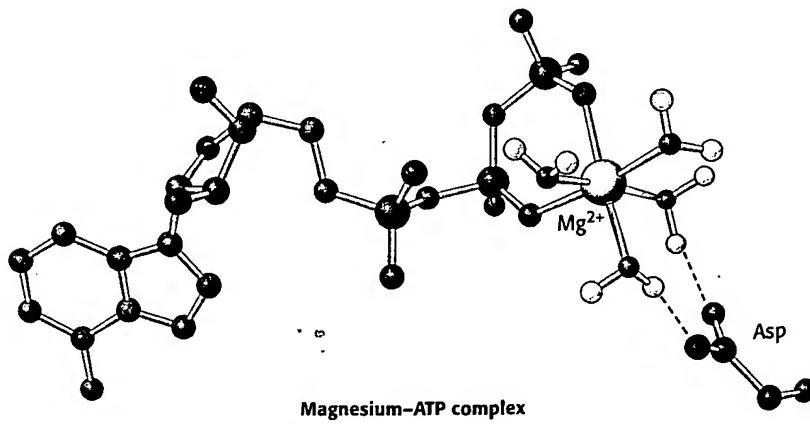


FIGURE 9.49 The structures of two isomeric forms of the ATP– Mg^{2+} complex. Other groups coordinated to the magnesium ion have been omitted for clarity.

FIGURE 9.50 ATP– Mg^{2+} complex bound to adenylate kinase. The magnesium ion is bound to the β and γ phosphoryl groups, and the four water molecules bound to the remaining coordination positions interact with groups on the enzyme, including a conserved aspartate residue. Other interactions have been omitted for clarity.



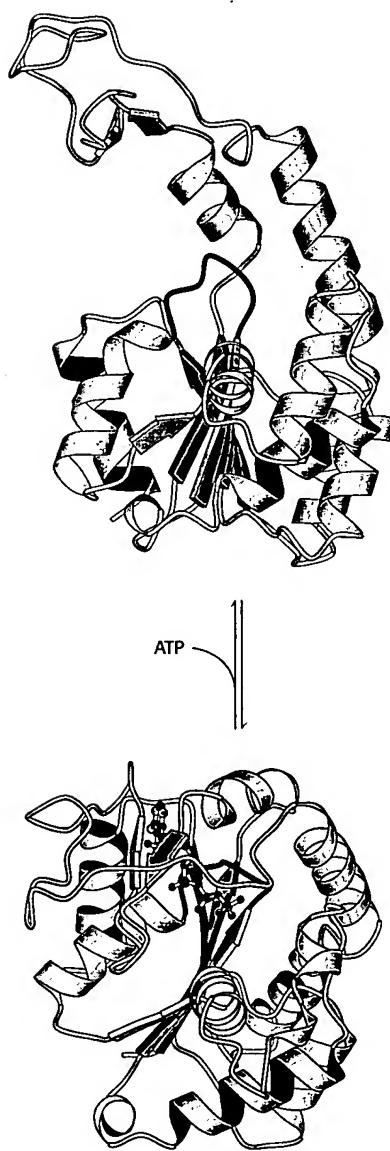
by water molecules. Oxygen atoms of the α and β , β and γ , or α and γ phosphoryl groups may contribute, depending on the particular enzyme. In addition, different stereoisomers are produced, depending on exactly which oxygen atoms bind to the metal ion. Third, the magnesium ion provides additional points of interaction between the ATP- Mg^{2+} complex and the enzyme, thus increasing the binding energy. In some cases, such as the DNA polymerases (Section 27.2.2), side chains (often aspartate and glutamate residues) of the enzyme can bind directly to the magnesium ion. In other cases, the enzyme interacts indirectly with the magnesium ion through hydrogen bonds to the coordinated water molecules (Figure 9.50). Such interactions have been observed in adenylate kinases bound to ATP analogs.

9.4.3 ATP Binding Induces Large Conformational Changes

Comparison of the structure of adenylate kinase in the presence and absence of an ATP analog reveals that substrate binding induces large structural changes in the kinase, providing a classic example of the use of induced fit (Figure 9.51). The P-loop closes down on top of the polyphosphate chain, interacting most extensively with the β phosphoryl group. The movement of the P-loop permits the top domain of the enzyme to move down to form a lid over the bound nucleotide. This motion is favored by interactions between basic residues (conserved among the NMP kinases), the peptide backbone NH groups, and the nucleotide. With the ATP nucleotide held in this position, its γ phosphoryl group is positioned next to the binding site for the second substrate, NMP. In sum, the direct interactions with the nucleotide substrate lead to local structural rearrangements (movement of the P-loop) within the enzyme, which in turn allow more extensive changes (the closing down of the top domain) to take place. The binding of the second substrate, NMP, induces additional conformational changes. Both sets of changes ensure that a catalytically competent conformation is formed only when both the donor and the acceptor are bound, preventing wasteful transfer of the phosphoryl group to water. The enzyme holds its two substrates close together and appropriately oriented to stabilize the transition state that leads to the transfer of a phosphoryl group from the ATP to the NMP. This is an example of *catalysis by approximation*. We will see such examples of a catalytically competent active site being generated only on substrate binding many times in our study of biochemistry.

9.4.4 P-Loop NTPase Domains Are Present in a Range of Important Proteins

 Domains similar (and almost certainly homologous) to those found in NMP kinases are present in a remarkably wide array of proteins, many of which participate in essential biochemical processes. Examples include ATP synthase, the key enzyme responsible for ATP generation; molecular motor proteins such as myosin; signal-transduction proteins such as transducin; proteins essential for translating mRNA into proteins, such as elongation factor Tu; and DNA and RNA unwinding helicases. The wide utility of P-loop NTPase domains is perhaps best explained by their ability to undergo substantial conformational changes on nucleoside triphosphate binding and hydrolysis. We shall encounter these domains (hereafter referred to as P-loop NTPases) throughout the book and shall observe how they function as springs, motors, and clocks. To allow easy recognition of these domains, they, like the binding domains of the NMP kinases, will be depicted with the inner surfaces of the ribbons in a ribbon diagram shown in purple and the P-loop shown in green (Figure 9.52).



 **FIGURE 9.51** Conformational changes in adenylate kinase. Large conformational changes are associated with the binding of ATP by adenylate kinase. The P-loop is shown in green in each structure. The lid domain is highlighted in yellow.

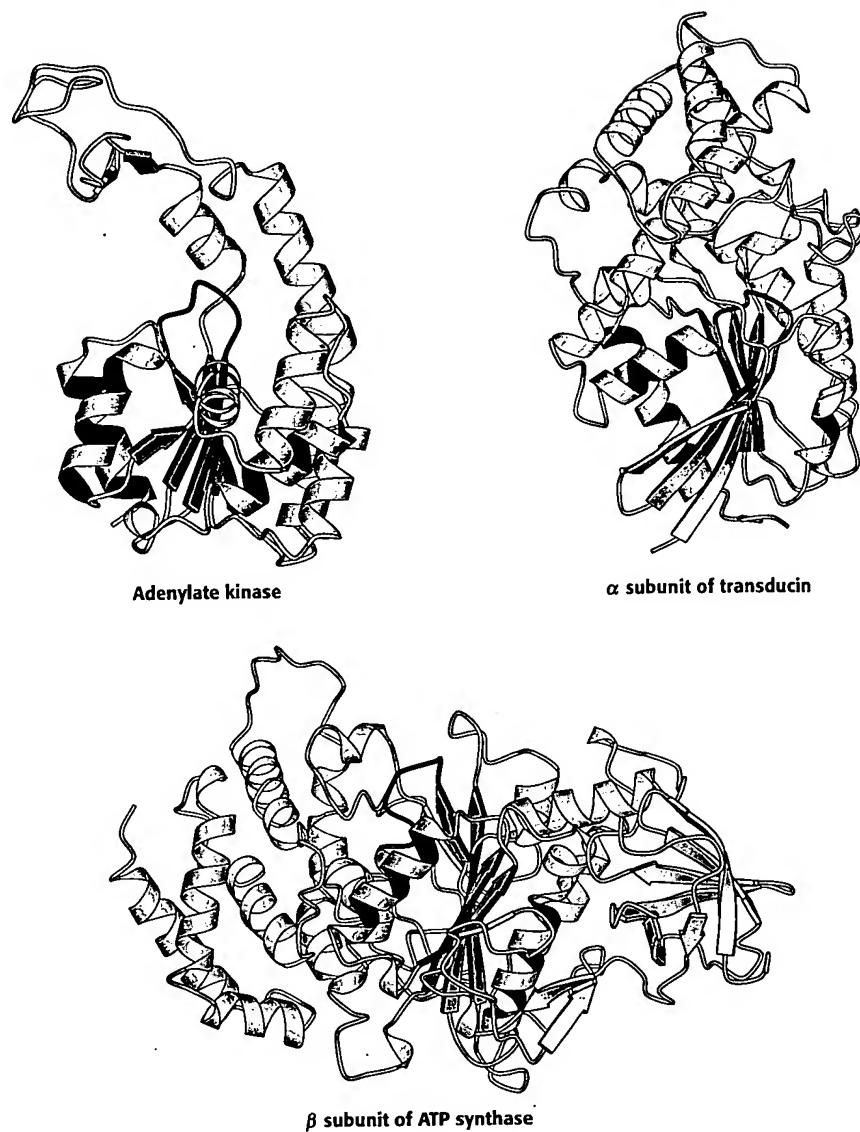


FIGURE 9.52 Three proteins containing P-loop NTPase domains. For the conserved domain, the inner surfaces of the ribbons are purple and the P-loops are green.

SUMMARY

Enzymes adopt conformations that are structurally and chemically complementary to the transition states of the reactions that they catalyze. Sets of interacting amino acid residues make up sites with the special structural and chemical properties necessary to stabilize the transition state. Enzymes use five basic strategies to form and stabilize the transition state: (1) the use of binding energy, (2) covalent catalysis, (3) general acid-base catalysis, (4) metal ion catalysis, and (5) catalysis by approximation. Of the enzymes examined in this chapter, three groups of enzymes catalyze the addition of water to their substrates but have different requirements for catalytic speed and specificity, and a fourth group of enzymes must prevent reaction with water.

• Proteases: Facilitating a Difficult Reaction

The cleavage of peptide bonds by chymotrypsin is initiated by the attack of a serine residue on the peptide carbonyl group. The attacking hydroxyl group is activated by interaction with the imidazole group of a histidine residue, which is, in turn, linked to an aspartate residue. This Ser-His-Asp catalytic triad generates a powerful nucleophile. The product of this

initial reaction is a covalent intermediate formed by the enzyme and an acyl group derived from the bound substrate. The hydrolysis of this acyl-enzyme intermediate completes the cleavage process. The tetrahedral intermediates for these reactions have a negative charge on the peptide carbonyl oxygen atom. This negative charge is stabilized by interactions with peptide NH groups in a region on the enzyme termed the oxyanion hole.

Other proteases employ the same catalytic strategy. Some of these proteases, such as trypsin and elastase, are homologs of chymotrypsin. In other proteases, such as subtilisin, a very similar catalytic triad has arisen by convergent evolution. Active-site structures that differ from the catalytic triad are present in a number of other classes of proteases. These classes employ a range of catalytic strategies but, in each case, a nucleophile is generated that is sufficiently powerful to attack the peptide carbonyl group. In some enzymes, the nucleophile is derived from a side chain; whereas, in others, an activated water molecule attacks the peptide carbonyl directly.

Carbonic Anhydrases: Making a Fast Reaction Faster

Carbonic anhydrases catalyze the reaction of water with carbon dioxide to generate carbonic acid. The catalysis can be extremely fast: molecules of some carbonic anhydrases hydrate carbon dioxide at rates as high as 1 million times per second. A tightly bound zinc ion is a crucial component of the active sites of these enzymes. Each zinc ion binds a water molecule and promotes its deprotonation to generate a hydroxide ion at neutral pH. This hydroxide attacks carbon dioxide to form bicarbonate ion, HCO_3^- . Because of the physiological roles of carbon dioxide and bicarbonate ions, speed is of the essence for this enzyme. To overcome limitations imposed by the rate of proton transfer from the zinc-bound water molecule, the most active carbonic anhydrases have evolved a proton shuttle to transfer protons to a buffer.

Restriction Enzymes: Performing Highly Specific DNA Cleavage Reactions

A high level of substrate specificity is often the key to biological function. Restriction endonucleases that cleave DNA at specific recognition sequences discriminate between molecules that contain these recognition sequences and those that do not. Within the enzyme-substrate complex, the DNA substrate is distorted in a manner that generates a magnesium ion binding site between the enzyme and DNA. The magnesium ion binds and activates a water molecule, which attacks the phosphodiester backbone.

Some enzymes discriminate between potential substrates by binding them with different affinities. Others may bind many potential substrates but promote chemical reactions efficiently only on specific molecules. Restriction endonucleases such as *EcoRV* endonuclease employ the latter mechanism to achieve levels of discrimination as high as million-fold. Structural studies reveal that these enzymes may bind nonspecific DNA molecules, but such molecules are not distorted in a manner that allows magnesium ion binding and, hence, catalysis. Restriction enzymes are prevented from acting on the DNA of a host cell by the methylation of key sites within their recognition sequences. The added methyl groups block specific interactions between the enzymes and the DNA such that the distortion necessary for cleavage does not take place.

Nucleoside Monophosphate Kinases: Catalyzing Phosphoryl Group Exchange Without Promoting Hydrolysis

Finally, NMP kinases illustrate that induced fit—the alteration of enzyme structure on substrate binding—facilitates phosphoryl transfer between nucleotides rather than to a molecule of water. This class of

enzyme displays a structural motif called the P-loop NTPase domain that is present in a wide array of nucleotide-binding proteins. The closing of the P-loop over a bound nucleoside triphosphate substrate permits the top domain of the enzyme to form a lid over the bound nucleotide, positioning the triphosphate near the monophosphate with which it will react, in an example of catalysis by approximation. These enzymes are dependent on metal ions, but the ions bind to substrate instead of directly to the enzyme. The binding of the metal ion to the nucleoside triphosphate enhances the specificity of the enzyme–substrate interactions by holding the nucleotide in a well-defined conformation and providing additional points of interaction, thus increasing binding energy.

KEY TERMS

binding energy (p. 228)
induced fit (p. 228)
covalent catalysis (p. 228)
general acid–base catalysis (p. 228)
metal ion catalysis (p. 228)
catalysis by approximation (p. 228)

chemical modification reaction (p. 230)
catalytic triad (p. 231)
oxyanion hole (p. 233)
protease inhibitor (p. 238)
proton shuttle (p. 243)
recognition sequence (p. 245)

restriction-modification system (p. 245)
in-line displacement (p. 246)
horizontal gene transfer (p. 252)
P-loop (p. 253)

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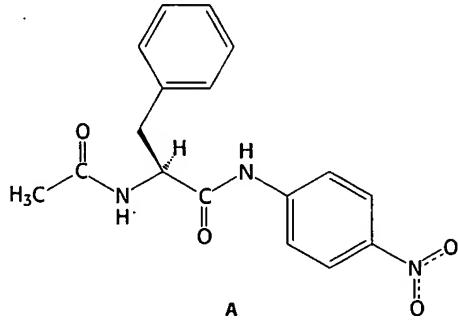
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PROBLEMS

1. *No burst.* Examination of the cleavage of the chromogenic amide substrate, A, by chymotrypsin with the use of stopped-flow kinetic methods reveals no burst. Why?



2. *Contributing to your own demise.* Consider the subtilisin substrates A and B.



These substrates are cleaved (between Phe and X) by native subtilisin at essentially the same rate. However, the His 64-to-Ala mutant of subtilisin cleaves substrate B more than 1000-fold as rapidly as it cleaves substrate A. Propose an explanation.

3. $1 + 1 \neq 2$. Consider the following argument. In subtilisin, mutation of Ser 221 to Ala results in a 10^6 -fold decrease in activity. Mutation of His 64 to Ala results in a similar 10^6 -fold de-

crease. Therefore, simultaneous mutation of Ser 221 to Ala and His 64 to Ala should result in a $10^6 \times 10^6 = 10^{12}$ -fold reduction in activity. Is this correct? Why or why not?

4. *Adding a charge.* In chymotrypsin, a mutant was constructed with Ser 189, which is in the bottom of the substrate specificity pocket, changed to Asp. What effect would you predict for this Ser 189 \rightarrow Asp 189 mutation?

5. *Conditional results.* In carbonic anhydrase II, mutation of the proton-shuttle residue His 64 to Ala was expected to result in a decrease in the maximal catalytic rate. However, in buffers such as imidazole with relatively small molecular components, no rate reduction was observed. In buffers with larger molecular components, significant rate reductions were observed. Propose an explanation.

6. *How many sites?* A researcher has isolated a restriction endonuclease that cleaves at only one particular 10-base-pair site. Would this enzyme be useful in protecting cells from viral infections, given that a typical viral genome is 50,000 base pairs long? Explain.

7. *Is faster better?* Restriction endonucleases are, in general, quite slow enzymes with typical turnover numbers of 1 s^{-1} . Suppose that endonucleases were faster with turnover numbers similar to those for carbonic anhydrase (10^6 s^{-1}). Would this increased rate be beneficial to host cells, assuming that the fast enzymes have similar levels of specificity?

8. *Adopting a new gene.* Suppose that one species of bacteria obtained one gene encoding a restriction endonuclease by horizontal gene transfer. Would you expect this acquisition to be beneficial?

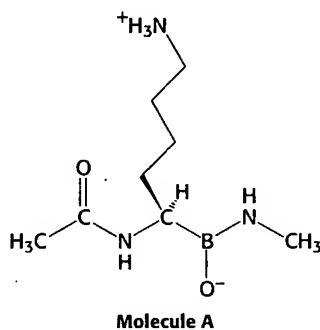
9. *Predict the product.* Adenylate kinase is treated with adenosine disphosphate (ADP).

(a) What products will be generated?

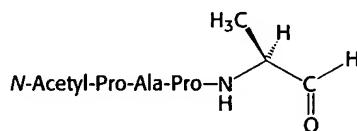
(b) If the initial concentration of ADP is 1 mM, estimate the concentrations of ADP and the products from part a after incubation with adenylate kinase for a long time.

10. *Chelation therapy.* Treatment of carbonic anhydrase with high concentrations of the metal chelator EDTA (ethylenediaminetetraacetic acid) results in the loss of enzyme activity. Propose an explanation.

11. *Identify the enzyme.* Consider the structure of molecule A. Which enzyme discussed in this chapter do you think molecule A will most effectively inhibit?



12. *An aldehyde inhibitor.* Elastase is specifically inhibited by an aldehyde derivative of one of its substrates:



(a) Which residue in the active site of elastase is most likely to form a covalent bond with this aldehyde?
 (b) What type of covalent link would be formed?

Mechanism Problem

13. *Complete the mechanism.* On the basis of the information provided in Figure 9.18, complete the mechanisms for peptide bond cleavage by (a) a cysteine protease, (b) an aspartyl protease, and (c) a metalloprotease.

Media Problems

14. *Now you see it, now you don't.* Pre-steady-state experiments using chymotrypsin and a chromogenic substrate (*N*-acetyl-L-phenylalanine *p*-nitrophenyl ester) show a "burst" of product at very short times (Figure 9.4). The **Conceptual Insights** module on enzyme kinetics explains this result. What results would you see if the product detected by the assay was the free N-terminal component of the substrate instead of the C-terminal component? (Hint: Use the pre-steady-state reaction simulation to simulate the experiment. Select different times following mixing and observe the amount of each product.).

15. *Seeing is disbelieving.* DIPF reacts specifically with serine 198 of chymotrypsin. One hypothesis as to why this is so might be that serine 198 is unusually exposed on the surface of the protein compared to other serines. After looking at the **Structural Insights** module on chymotrypsin, what do you think of this hypothesis?

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